Biochemical Pharmacology, Vol. 52, pp. 1295–1303, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
PII S0006-2952(96)00485-6

Biological Properties of Fluoroglutamate-Containing Analogs of Folates and Methotrexate with Altered Capacities to Form Poly (γ-glutamate) Metabolites

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ABSTRACT. Fluoroglutamate-containing analogs of folates and methotrexate (MTX) with altered capacities for poly (γ-glutamate) metabolism were synthesized to probe the biological roles of polyglutamates. Compared to folic acid, DL-*e*,*t*-γ-fluorofolic acid, a compound that is a poor substrate for polyglutamylation, was ≈25-fold less potent in promoting growth of folate-depleted H35 rat hepatoma cells. DL-β,β-Difluorofolic acid, a compound that forms diglutamates more readily than does folic acid, was at least equivalent to folic acid in potency. Leucovorin (LV), a reduced folate, was 30-fold more potent than folic acid in promoting growth, whereas the analogous form of DL-*e*,*t*-γ-fluorofolate, DL-*e*,*t*-γ-fluoroleucovorin (DL-*e*,*t*-γ-FLV) was only 4-fold more potent than folic acid. Both LV and DL-*e*,*t*-γ-FLV protected or "rescued" cells from the growth inhibitory effects of MTX; however a 37- to 46-fold higher concentration of the fluoro analog was required. Folic acid, DL-*e*,*t*-γ-fluorofolic acid, LV, and DL-*e*,*t*-γ-FLV each potentiated the growth inhibitory effect of 5-fluoro-2′-deoxyuridine on CCRF-CEM human leukemia cells; higher concentrations of fluorinated analogs again were required. Stereochemically pure L-*t*-γ-fluoromethotrexate (L-*t*-γ-FMTX), a poor substrate for polyglutamylation, was evaluated as a cell growth inhibitor. In continuous exposure, L-*t*-γ-FMTX was 7-fold less potent than MTX as an inhibitor of CCRF-CEM growth. Results with these fluorinated folate and MTX analogs offer insight into the importance of polyglutamate metabolism to these biological and pharmacological effects. BIOCHEM PHARMACOL 52;8:1295–1303, 1996.

KEY WORDS. polyglutamate; folylpolyglutamate synthetase; leucovorin; 4-fluoroglutamate; 3,3-difluoroglutamate; folic acid; methotrexate

Folates are essential vitamins in human nutrition [1]. Poly (γ-glutamate) metabolites of folates are the active intracellular cofactors for folate-dependent enzymes and allow retention of folate pools far above their extracellular concentration [2]. An absolute requirement for folylpolyglutamates for cell growth and viability was first demonstrated in a classic study using polyglutamylation-deficient mutants of Chinese hamster ovary cells [3].

In addition to their nutritional role, folates are used at pharmacological doses in two cancer chemotherapy regimens: LV§ "rescue" of MTX toxicity [4] and potentiation of the cytotoxicity of fluoropyrimidines by folic acid and reduced folates [5, 6]. The requirement for and role of folylpolyglutamates in producing these interactions *in vitro* and in producing therapeutically selective interactions *in vivo* are not defined as clearly as is their requirement in nutrition.

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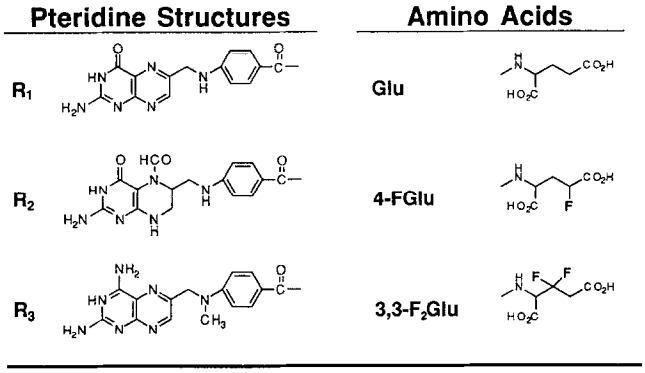
§ *Abbreviations:* 3,3-F_2Glu, 3,3-difluoroglutamic acid; 4-FGlu, 4-fluoroglutamic acid; EC_{50}, drug concentration effective in inhibiting growth to 50% of untreated control; (6S)-DL-*e*,*t*-γ-FLV, (6S)-DL-*e*,*t*-γ-fluoroleucovorin [(6S)-5-formyl-5,6,7,8-tetrahydropteroyl (DL-*e*,*t*-4-fluoroglutamic acid)]; (6*RS*)-L-*t*-γ-FLV, (6*RS*)-L-*t*-γ-fluoroleucovorin [(6*RS*)-5-formyl-5,6,7,8-tetrahydropteroyl (L-*t*-4-fluoroglutamic acid)]; L-*t*-γ-FMTX, L-*t*-γ-fluoromethotrexate [4-amino-10-methylpteroyl (L-*t*-4-fluoroglutamic acid)]; FPGS, folylpolyglutamate synthetase; 5-FUdR, 5-fluoro-2′-deoxyuridine; G_{50} and G_{90}, concentrations supporting 50 and 90%, respectively, of maximal growth; LV, leucovorin (5-formyl-5,6,7,8-tetrahydrofolate); MTX, methotrexate (4-amino-10-methylpteroylglutamic acid); DL-*e*,*t*-γ-PteFGlu, DL-*e*,*t*-γ-fluorofolic acid [pteroyl (DL-*e*,*t*-4-fluoroglutamic acid)]; DL-*e*,*t*-γ-H_2PteFGlu, DL-*e*,*t*-γ-dihydrofluorofolic acid, [7,8-dihydropteroyl (DL-*e*,*t*-4-fluoroglutamic acid)]; (6*R*)-10-HCO-DL-*e*,*t*-γ-H_4PteFGlu, (6*R*)-10-formyl-DL-*e*,*t*-γ-tetrahydrofluorofolic acid, [(6*R*)-10-formyl-5,6,7,8-tetrahydropteroyl (DL-*e*,*t*-4-fluoroglutamic acid)]; DL-β,β-PteF$_2$Glu, pteroyl (DL-3,3-difluoroglutamic acid); PteGlu, folic acid (pteroylglutamic acid); and TS, thymidylate synthase.

Received 14 February 1996;accepted 14 May 1996.

One general approach to studying the requirement for folate polyglutamylation in nutrition and chemotherapeutic regimens is to use conservative structural analogs that retain the biochemical properties of folate monoglutamates, with the exception that the ability of the analog to form polyglutamates is either decreased or increased. Comparison of the *in vitro* and *in vivo* effects of a folate and its polyglutamylation-modified analog in growth promotion and in each chemotherapeutic regimen should allow a direct measure of the importance of polyglutamylation since other changes in cellular pharmacology would be eliminated. A clear advantage of a general chemical approach to the study of polyglutamylation over a genetic approach is that it can be readily used in diverse cell lines, as well as *in vivo*. With respect to chemotherapeutic studies, a further advantage is that the chemical approach can be used in cells possessing a full complement of folylpolyglutamates at the start of treatment, as would occur *in vivo*.

This general chemical approach requires analogs that are altered in polyglutamylation, but not in other biochemical properties. We have implemented this approach by substituting either 4-FGlu or 3,3-F_2Glu for Glu in folates and antifolate analogs (Fig. 1).* Analogs of folates [9] or antifolates [8–11] containing 4-FGlu have a dramatically reduced capacity for polyglutamylation by purified FPGS, while analogs of folates or antifolates containing 3,3-F_2Glu form diglutamates with significantly higher efficiency than do natural folates [11]. These data suggested that intracellular polyglutamylation of 4-FGlu-containing species would be greatly reduced; this hypothesis was confirmed for the 4-FGlu-containing analog of MTX [8]. Fluorination might not affect other biochemical properties of the analogs significantly, despite the known electronic effects of fluorine, since fluorine and hydrogen are very similar with respect to their atomic radii [12]. Studies of the 4-FGlu-containing analog of MTX [8–11, 13] with purified folate-dependent enzymes and in intact cells suggested that other biochemical interactions were affected minimally. Despite some limitations imposed by the fact that the analogs containing 4-FGlu can be polyglutamylated at a very low slow rate and that there are some changes in other biochemical properties, these analogs can still be useful for assessing whether polyglutamates are rate-limiting to particular cellular processes.

Analogs of folic acid [9] and LV containing 4-FGlu as well as the folic acid analog containing 3,3-F_2Glu [11] have been synthesized (Fig. 1). The effects of these analogs as growth factors for cells, their ability to participate in protection and "rescue" from MTX toxicity, and potentiation of growth inhibitory potency of 5-FUdR have been evaluated. In addition, stereochemically pure L-*t*-γ-FMTX [9], a poorly polyglutamylatable MTX analog, has been characterized with respect to its growth inhibitory potency and its ability to be protected by LV.



Folate or Analog	Structure
PteGlu	R_1-Glu
DL-*e,t*-γ-PteFGlu	R_1-4-FGlu
DL-β,β-PteF_2Glu	R_1-3,3-F2Glu
LV	R_2-Glu
DL-*e,t*-FLV	R_2-4-FGlu
L-*t*-FLV	R_2-4-FGlu
MTX	R_3-Glu
L-*t*-γ-FMTX	R_3-4-FGlu

FIG. 1. Structures of folic acid (PteGlu), leucovorin (LV), methotrexate (MTX), and their fluorinated analogs.

MATERIALS AND METHODS

Materials

MTX and (6*RS*)- and (6*S*)-LV were gifts of Immunex (Seattle, WA) and Glaxo Wellcome (Research Triangle Park, NC), respectively. Pteridine solutions were standardized using extinction coefficients for MTX or appropriate folate [14]. 2-Mercaptoethanol was from Eastman Kodak (Rochester, NY). 5-FUdR was from Sigma (St. Louis, MO). Common chemicals were reagent grade or higher.

Synthesis of Folic Acid and MTX Analogs

DL-*e,t*-γ-PteFGlu, L-*t*-γ-PteFGlu, DL-ββ,-PteF_2Glu, and L-*t*-γ-FMTX were synthesized, purified, and chemically characterized as described [9, 11].

Synthesis of (6RS) -L-t-γ-FLV

The general method of Moran and Coleman [15] was used. To a solution of L-*t*-γ-PteFGlu (64 mg; 0.139 mmol) in

* The designations L- and DL- refer to the chirality of the α-carbon of the free amino acid (e.g., L-Glu refers to L-glutamic acid) or of the amino acid residue incorporated into a folate/antifolate structure [e.g. L-PteGlu refers to Pte (L-Glu)]. The designations *erythro* and *threo* refer to the relative stereochemistry of the α and γ positions in the free amino acid (e.g., L-*threo*-4-fluoroglutamate is (2S,4S)-4-fluoroglutamate [7]) or of the amino acid residue incorporated into a folate/antifolate structure (e.g. L-*threo*-γ-PteFGlu refers to Pte [2S,4S)-4-FGlu]; [8]).

water (10 mL) was added $NaBH_4$ (65 mg; 1.71 mmol); after stirring at room temperature for 16 hr, additional $NaBH_4$ (25 mg) was added, and the solution was stirred for 2 hr. 2-Mercaptoethanol (50 μL) was added, and the pH was lowered to 6.0 with dilute HCl; an additional 50 μL of 2-mercaptoethanol was then added. Formic acid (0.77 mL; 15.9 mmol) was added to the solution, which lowered the pH to 2. Dilute NaOH was used to raise the pH to 3.8 and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (41 mg; 0.21 mmol) was added to the solution. The mixture was stirred at room temperature for 30 min and then evaporated to dryness. The resulting solid was purified by DEAE-cellulose chromatography (20 g Whatman DE-53; gradient from 0.3–1.0 M ammonium acetate, pH 7.0). Fractions containing pure (6*RS*)-*L*-*t*-γ-FLV were identified by their UV spectra, pooled, and lyophilized to yield the acetate salt (0.011 mmol; 8%). ^{1}H NMR (360 MHz, D_2O) (2 rotamers) δ 8.41 and 7.7 (2s, 1 H), 7.41 and 7.43 (d, 2 H), 6.46 and 6.50 (d, 2 H), 4.42 (m, 1 H), 3.41–2.88 (m, 6 H), 2.29–2.12 (m, 2 H), 1.8 (s, acetate); ^{13}C NMR (90.5 MHz, D_2O) δ 177.1, 176.9, 170.1, 164.5 and 162.5, 158.6, 153.5, 152.7, 151.5 and 151.3, 129.2 (2C), 120.8 and 121.0, 112.3 and 112.1 (2C), 89.2, 88.6 (d, J = 246.6 Hz), 51.2, 49.4, 42.9 and 43.3, 41.6 and 41.8, 34.8 (d, J = 20.3 Hz); ^{19}F NMR (470 MHz, D_2O) −106.3 (bm); MS (FAB$^-$, triethanolamine) for $C_{20}H_{22}FN_7O_7$ m/z (relative intensity) 490 ((M-H)$^-$, 70); (FAB$^+$, dithiothreitol/dithioerythritol) m/z (relative intensity) 492 ((M + H)$^+$, 49.3); HRMS (FAB$^+$, dithiothreitol/dithioerythritol) for $C_{20}H_{22}FN_7O_7$ (M + H)$^+$ calcd 492.1643, found 492.1662.

Synthesis of (6S)-DL-e,t-γ-FLV

DL-*e*,*t*-γ-PteFGlu (268 μmol in 15 mL H_2O) was added to 58.7 mL water mixed with 6.7 mL of 10% (w/v) Na_2CO_3 and 53.6 mL of 20% (w/v) sodium-ascorbate, pH 6.0 [16]. After mixing at room temperature (final pH = 9.78), 2.68 g of $Na_2O_4S_2$ was added and mixed for 5 min. The solution (final pH = 8.84) was cooled to 0° and DL-*e*,*t*-γ-H_2PteFGlu was precipitated by slow (40 min) addition of 5 N HCl to pH 1.9. Twenty minutes after the last addition, the solution was centrifuged (1900 *g*, 10 min, 4°), and the pellet was washed with 3 × 90 mL of iced 10 mN HCl to yield 215 μmol (80% yield) DL-*e*,*t*-γ-H_2PteFGlu. UV-visible spectra of this material at pH 1 and 13 matched those of dihydrofolate. DL-*e*,*t*-γ-H_2PteFGlu was converted to (6*R*)-10-HCO-DL-*e*,*t*-γ-H_4PteFGlu by coupled dihydrofolate reductase and 10-formyl-tetrahydrofolate synthetase activities. The final reaction mixture (500 mL; 37°) contained 430 μM DL-*e*,*t*-γ-H_2PteFGlu, 25 mM Tris-HCl (pH 7.0), 1 mM ATP, 10 mM $MgCl_2$, 25 mM potassium-formate, 50 mM 2-mercaptoethanol, 16 IU *Lactobacillus casei* dihydrofolate reductase (Biopure, Boston, MA), 100 IU of *Clostridium acidi-urici* 10-formyl-tetrahydrofolate synthetase ([17]; a gift of Dr. J. C. Rabinowitz, University of California, Berkeley) and an NADPH-regenerating system [500 units of glucose-6-phosphate dehydrogenase (Torula yeast, type XII; Sigma), 0.4 mM glucose-6-phosphate (Sigma), and 25 μM NADPH (Boehringer-Mannheim, Indianapolis, IN)]. Components were degassed prior to mixing and bubbled with N_2 prior to addition of enzymes; after addition of enzymes the mixture was incubated at 37°, protected from light. Formation of (6*R*)-10-HCO-DL-*e*,*t*-γ-H_4PteFGlu was followed by acidification of aliquots and measuring absorbance at 350 nm after ≥10 min to determine the concentration of 5,10-methenyl species [17]; appropriate reactions lacking enzymes were used as control. After <60 min, the reaction reached completion (109 μmol; 55% yield). Protein, which interfered with subsequent chromatography, was removed by ultrafiltration at 4° (Amicon PM-30, Beverly, MA). The ultrafiltrate was applied at 4° to a DEAE-cellulose column (2 × 40 cm; DE-23, Whatman, Inc., Clifton, NJ) equilibrated with 50 mM ammonium acetate, pH 8.0, containing 50 mM 2-mercaptoethanol (redistilled to remove nonlyophilizable contaminants). After sample application, the column was washed with 150 mL of equilibration buffer; then a gradient (750 mL total volume) from 50–250 mM ammonium acetate, pH 8.0, containing 50 mM redistilled 2-mercaptoethanol was developed, followed by a 500-mL wash with 250 mM buffer [17]. Spectra (230–370 nm) of fractions and the absorbance at 350 nm of acidified (above) 25-μL samples showed that (6*R*)-10-HCO-DL-*e*,*t*-γ-H_4PteFGlu eluted at the end of the gradient; HPLC analysis indicated contamination with ADP and ATP, but not with NADP(H). The pooled material (103 μmol) was lyophilized. During lyophilization, the pH decreased and the product was converted to its more stable 5,10-methenyl form. This dried material was dissolved in 50 mM 2-mercaptoethanol (redistilled) and relyophilized. The final yellow powder was dissolved at 10 mM in 1% (w/v) sodium-ascorbate, and the solution was adjusted to pH 7.0 with 1 N NaOH. The sample was then autoclaved (120°, 15 psi) for 30 min followed by a 20-min slow exhaust. After clarification by centrifugation (1900 *g*, 10 min, 4°), the sample was loaded at 4° onto a DEAE-cellulose column (2 × 40 cm; DE-23) equilibrated with 125 mM ammonium acetate, pH 8.0 (no 2-mercaptoethanol). The column was eluted successively with 125 mM ammonium acetate (300 mL), a linear gradient from 125–500 mM ammonium acetate, pH 8.0 (500 mL total volume), and 500 mM ammonium acetate, pH 8.0 (250 mL). Fractions containing (6*S*)-DL-*e*,*t*-γ-FLV (identified by its UV spectrum) eluted at the start of the 500 mM wash, well separated from ascorbate and adenine nucleotides. Fractions were pooled and lyophilized to yield a whitish powder (60 μmol). Absorption spectra of the final material at pH 7 and 1 were identical to those published for LV and identical to those of an authentic sample of LV synthesized by the same procedure. Acidification of a pH 7 solution of (6*S*)-DL-*e*,*t*-γ-FLV led to the acid spectrum over a 60- to 90-min interval; overlapping spectra taken from 0–90 min showed sharp isosbestic points at 258 and 317 nm. The time–course and isosbestic points are typical for acidficiation of pure LV. HPLC analysis

showed that the final material is >98.8% pure and contains <0.06% LV (limit of detection).

HPLC Analysis

Analytical HPLC was performed on a Rainin Instruments HPLC system using the Dynamax controller and data capture module run on a Macintosh computer [18]. Eluant was monitored at 280 and 254 nm. Anion-exchange HPLC was performed on an Ultrasil AX column (0.4 × 25 cm; Beckman) at 45° eluted isocratically at 0.8 mL/min with 35–85 mM sodium-phosphate, pH 3.3; the concentration required to obtain a constant elution time (7.5 min) for MTX depended on column age. Each analog was >98% pure based on the area detected in HPLC.

Cell Lines

Human T-lymphoblastic leukemia cell line CCRF-CEM [19] and subline R30dm [20] were cultured as described [20]. R30dm is resistant to intermittent MTX exposure as a result of decreased MTX polyglutamylation; R30dm has 1% of the FPGS specific activity (measured with MTX) of CCRF-CEM. The H35 rat hepatoma cell line was cultured as described [10]. Folate-depleted H35 cells were produced by growth of H35 in folate-free Swim's medium containing 10% undialyzed fetal bovine serum for 1 week prior to use. Using the GenProbe test kit, all cell lines were negative for Mycoplasma contamination.

Promotion or Inhibition of Growth of Cell Lines In Vitro

Growth promotion of folate-depleted H35 cells was assayed by adding folates or analogs to folate-free Swim's medium containing 10% undialyzed fetal bovine serum and measuring growth after 72 hr. G_{50} or EC_{50} values were determined from plots of percent control growth versus the logarithm of drug concentration.

Growth inhibition of H35 cells in continuous (48 hr) and intermittent drug exposures was measured as described [10]. Growth inhibition of CCRF-CEM and R30dm in continuous (120 hr) and intermittent drug exposures was measured as described [20]. When 24-hr drug exposure of CCRF-CEM was used, drug was present from 0 to 24 hr. Then cells were centrifuged (5 min, 1000 *g*, ambient temperature), and the medium (5 mL) was aspirated and replaced with an equal volume of RPMI 1640 medium (37°). After re-centrifugation and aspiration of medium, cells were resuspended in 5 mL complete medium and incubated for the remainder of the 120-hr growth period. The wash was estimated to lower the drug concentration ≥625-fold to a level at which it had no effect during the remaining growth period.

Protection against growth inhibition was assayed by simultaneously adding LV or its fluorinated analogs [e.g. (6S)-DL-*e*,*t*-γ-FLV] with an MTX concentration previously shown to inhibit growth 80–97%; the remainder of the assay was as described above.

To assess "rescue" of MTX-induced growth inhibition, H35 cells were subcultured and, after 24 hr when logarithmic growth was re-established, the cells were exposed for 2 or 24 hr to MTX at concentrations previously shown to inhibit growth by 50 or 90%, respectively. After drug exposure, cells were washed three times with PBS, and medium containing LV (0.1 to 20 μM) or (6S)-DL-*e*,*t*-γ-FLV (1–200 μM) was added. Cell growth was measured 48 hr after the end of the drug exposure as described above.

Enzymes

TS was partially purified from CCRF-CEM cells and assayed as described [21].

RESULTS

Fluorinated Folate Analogs as Growth Factors for Folate-Depleted H35 Rat Hepatoma Cells

The ability of fluorinated folate analogs to replace folates as growth factors was assessed in short-term folate-depleted H35 cells. PteGlu supported half-maximal growth at 95 nM, while 400 nM was required for maximal growth (Table 1). LV, a reduced folate, was ≈30-fold more potent than PteGlu as a growth factor in this system, with a similarly shaped concentration–response curve (not shown). Each fluorinated folate analog also supported maximal growth of H35 cells (Table 1). The shapes of their concentration–response curves were essentially identical to those of PteGlu and LV (not shown); however the G_{50} depended on the position of fluorine substitution and also on the oxidation state of the pyrazine ring. The G_{50} and G_{90} for DL-*e*,*t*-γ-PteFGlu were 17- to 27-fold higher than for PteGlu; for DL-β,β-$PteF_2Glu$ both values were similar to those of PteGlu. The fluorinated analog of LV, (6S)-DL-*e*,*t*-γ-FLV, was 100-fold more potent than DL-*e*,*t*-γ-PteFGlu, but was still 10-fold less potent than LV itself. The LV

Table 1. Influence of folates and fluorofolates on the growth of folate-depleted (short-term) H35 rat hepatoma cells

Folate added	G_{50} (nM)	G_{90} (nM)
PteGlu	95 ± 11	398 ± 29
DL-*e*,*t*-γ-PteFGlu	2550 ± 310	6805 ± 800
(6S)-LV	3.0 ± 0.4	10.2 ± 0.8
(6S)-DL-*e*,*t*-γ-FLV	24.5 ± 3.1	78 ± 6.8
(6RS)-L-*t*-γ-FLV	41.8 ± 5.4	129 ± 14
DL-β,β-$PteF_2Glu$	63 ± 8	470 ± 50

H35 cells were depleted of folates by growth in folate-free Swim's medium containing 10% undialyzed fetal bovine serum for 1 week. Cells were inoculated at 1 × 10^4 cells/200 μL/well in 96-well plates in medium containing the indicated levels of folic acid or analog and counted 72 hr later. G_{50} and G_{90} are the concentrations required to support 50 or 90 % of maximal growth (i.e. growth at 4 μM PteGlu), respectively. Values are means ± SD (N = 6). Values are not corrected for the low level (11-15%) of growth in the absence of any addition. Concentrations listed represent the total for all isomers present in the sample; as discussed in the text, the D-enantiomers are probably inactive.

analog containing 3,3-F_2Glu is now being synthesized for separate study.

Between 2 and 25 μM DL-β,β-PteF_2Glu, growth inhibition occurred (Fig. 2), whereas no inhibition was seen with PteGlu at ≤25 μM (Fig. 2; data for 4–25 μM from separate experiments are not shown). Growth inhibition (92% inhibition at 25 μM) was completely protected against by the simultaneous presence of 0.1–10 μM (6S)-LV; partial protection occurred at as low as 1–2 nM (6S)-LV. The combination of thymidine (20 μM) and hypoxanthine (50 μM) also completely protected, but only partial protection was afforded by either agent alone. PteGlu at 50 μM protected only to a minor extent.

Protection from MTX-Induced Growth Inhibition

Folate-replete H35 cells that are inhibited by MTX (91% inhibition at 50 nM) were partially protected (50% growth inhibition) by 0.03 μM (6S)-LV, but a similar degree of protection required 1.1 μM (6S)-DL-*e,t*-γ-FLV; both compounds provided full protection at higher concentrations. At an MTX concentration that inhibited growth by 50%, a comparable difference in potency of (6S)-LV and (6S)-DL-*e,t*-γ-FLV was noted. Similar protection data were obtained in H35 cells with (6*RS*)-L-*t*-γ-FLV (not shown) and with both (6S)-DL-*e,t*-γ-FLV and (6*RS*)-L-*t*-γ-FLV in CCRF-CEM cells (not shown).

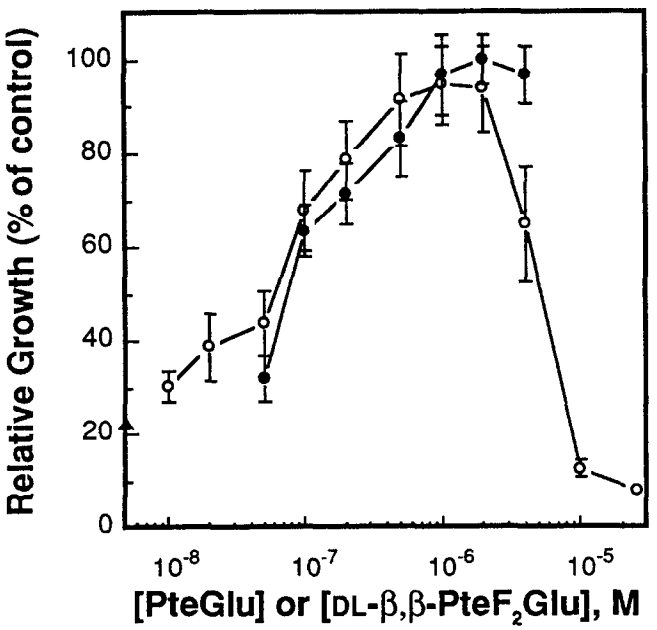


FIG. 2. Concentration-dependent effects of PteGlu and DL-β,β-PteF_2Glu on folate-depleted H35 rat hepatoma cells. H35 cells were depleted of folates for 1 week as described in Materials and Methods. Cells were then subcultured in 96-well plates at 5 × 10^3 cells/200 μL/well in folate-free medium without supplement (▲; on left axis) or supplemented with either PteGlu (●) or DL-β,β-PteF_2Glu (○), and cell growth was evaluated after 72 hr. Values are means ± SD (N = 6).

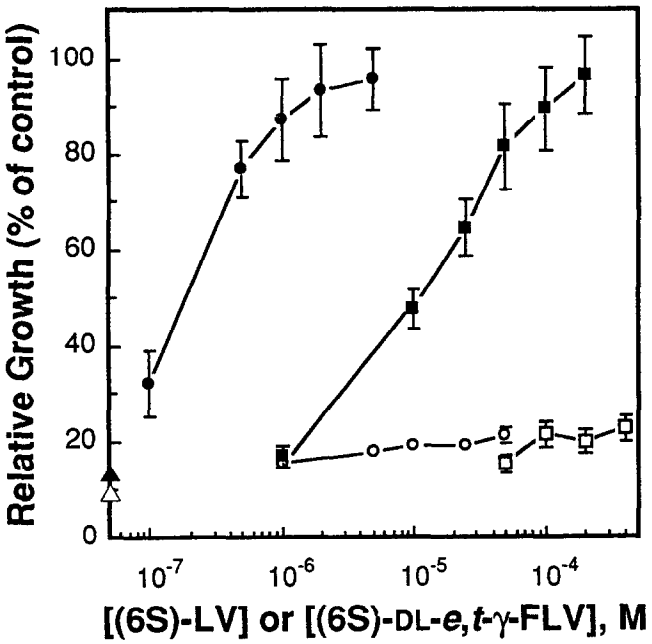


FIG. 3. "Rescue" by (6S)-LV or (6S)-DL-*e,t*-γ-FLV of H35 cell growth inhibition following a 2- or 24-hr exposure to MTX. H35 cells were subcultured in 96-well plates at 5 × 10^3 cells/200 μL/well, grown for 24 hr, and then exposed to either 10 μM MTX for 2 hr or to 0.2 μM MTX for 24 hr (an MTX concentration giving ≈90% growth inhibition at each exposure time). After the exposure, cells were washed twice to remove extracellular MTX, and growth was quantitated after 48 hr in the absence (▲, △; 2- and 24-hr MTX, respectively; on left axis) or presence of the indicated concentration of (6S)-LV (●, ○; 2- and 24-hr MTX, respectively) or (6S)-DL-*e,t*-γ-FLV (■, □; 2- and 24-hr MTX, respectively). Values are means ± SD (N = 6).

"Rescue" from MTX-Induced Growth Inhibition

After cells are exposed to MTX and drug is removed, LV may reverse the growth inhibition ("rescue"; [4]). Folate-replete H35 cells were exposed to MTX for either 2 or 24 hr, the drug was removed, and (6S)-LV or (6S)-DL-*e,t*-γ-FLV was present during the ensuing 48-hr growth period (Fig. 3). After a 2-hr MTX exposure, both agents completely rescued cells from MTX-induced growth inhibition; rescue to 50% growth inhibition from the control value of 87% growth inhibition required 0.19 μM (6S)-LV, but 11 μM (6S)-DL-*e,t*-γ-FLV. After a 24-hr exposure at an MTX concentration that achieved about the same degree of growth inhibition, neither (6S)-LV nor (6S)-DL-*e,t*-γ-FLV rescued cells (Fig. 3).

Enhancement of 5-FUdR Potency

PteGlu and LV can enhance the potency of 5-FUdR as a cell growth inhibitor [6, 22]; however, higher concentrations of PteGlu are required. The abilities of PteGlu and DL-*e,t*-γ-PteFGlu to potentiate growth inhibition of CCRF-CEM cells by 5-FUdR in continuous exposure were compared (Fig. 4). At high concentrations, PteGlu achieved

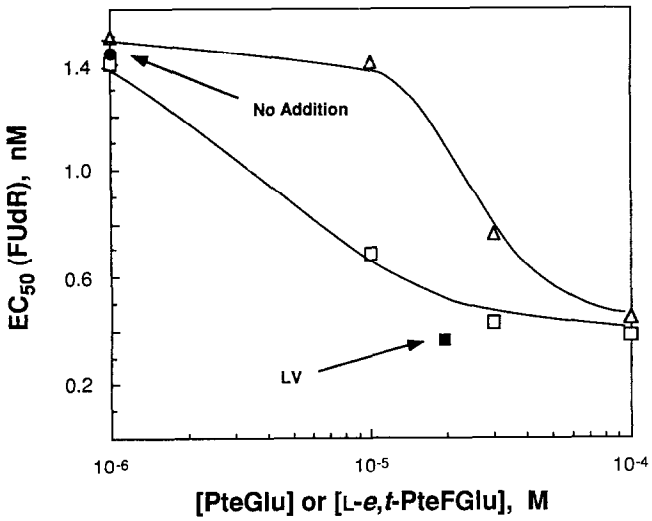


FIG. 4. Potentiation by PteGlu or DL-*e*,*t*-γ-PteFGlu of the potency of 5-FUdR as a growth inhibitor of CCRF-CEM cells during continuous exposure. The EC_{50} for inhibition of CCRF-CEM growth by 5-FUdR was determined as described in Materials and Methods in the absence of any addition to the medium (●), the presence of 20 μM (6*RS*)-LV (■), or the presence of the indicated concentration of PteGlu (□) or DL-*e*,*t*-γ-PteFGlu (△). The concentration of DL-*e*,*t*-γ-PteFGlu is given as the active L-*e*,*t*-isomers only.

the same degree of potentiation as that achievable by 20 μM (6RS)-LV, an optimal concentration [6]. DL-*e*,*t*-γ-PteFGlu was able to potentiate to the same degree as PteGlu, but higher concentrations were required; 20 μM (6S)-DL-*e*,*t*-γ-FLV could also fully potentiate (see below). Note that DL-*e*,*t*-γ-PteFGlu contained <0.2% PteGlu (limit of HPLC detection); thus, contamination cannot account for these results. The FPGS-deficient CCRF-CEM subline R30dm is slightly less sensitive to 5-FUdR in the absence of modulation [6]. The potency of continuous exposure of 5-FUdR against R30dm could also be potentiated by PteGlu (Fig. 5; [6]); however, DL-*e*,*t*-γ-PteFGlu could not potentiate the effects of 5-FUdR in this cell line. The latter result also reinforces the conclusion that the effects seen with CCRF-CEM (Fig. 4) cannot result from PteGlu contamination since in that case the response to DL-*e*,*t*-γ-PteFGlu should be the same in both cell lines.

The effect of drug exposure time was studied by exposing CCRF-CEM cells to 5-FUdR in the absence or presence of PteGlu or DL-*e*,*t*-γ-PteFGlu during only the first 24 hr of growth, after which both agents were removed and growth was continued in their absence for the remainder of the 120-hr growth period. Whether exposure time was for 120 hr (EC_{50} = 1.35 nM) or 24 hr (EC_{50} = 1.2 nM), there was no difference in the potency of 5-FUdR alone. The concentration-dependence of PteGlu enhancement of 5-FUdR potency (not shown) was similar to that seen when both were present continuously (Fig. 4). The concentration-dependence of potentiation by DL-*e*,*t*-γ-PteFGlu was also similar to that during continuous exposure; however, full potentiation was not reached even at 10^{-4} M. Since the EC_{50} was still decreasing at 10^{-4} M DL-*e*,*t*-γ-PteFGlu and was near maximum potentiation, higher concentrations of DL-*e*,*t*-γ-PteFGlu may be required at the 24-hr exposure time.

The abilities of the reduced folates (6*RS*)-LV and (6S)-DL-*e*,*t*-γ-FLV to enhance 5-FUdR potency were also assessed (data not shown). In CCRF-CEM cells, maximum potentiation occurred at ≥1 μM (6*RS*)-LV, with potentiation increasing most dramatically between 0.01 and 0.1 μM (data not shown and [6]). Potentiation by (6S)-DL-*e*,*t*-γ-FLV increased gradually over the range 0.2 to 20 μM. At 20 μM (6S)-DL-*e*,*t*-γ-FLV, potentiation was essentially the same as that achieved with 20 μM (6*RS*)-LV; however, at concentrations giving half-maximal potentiation, (6S)-DL-*e*,*t*-γ-FLV was ≈22-fold less effective than (6*RS*)-LV. The effect of (6*RS*)-L-*t*-γ-FLV paralleled that of (6S)-DL-*e*,*t*-γ-FLV. In one experiment with the FPGS-deficient subline R30dm, (6S)-LV enhanced 5-FUdR potency (as seen previously [6]), with maximum potentiation observed at 1 μM (6*RS*)-LV. In contrast, at concentrations up to 20 μM (6S)-DL-*e*,*t*-γ-FLV or 20 μM (6RS)-L-*t*-γ-FLV, a much smaller degree of potentiation was observed.

L-threo-FMTX Effects on Cell Growth and Protection by LV

L-*t*-γ-FMTX is a potent DHFR inhibitor (Table 2; [9], but is not a substrate for polyglutamylation [9]. Substitution by 4-FGlu in MTX did not appreciably enhance TS inhibition (Table 2), and thus DHFR remains the primary target of

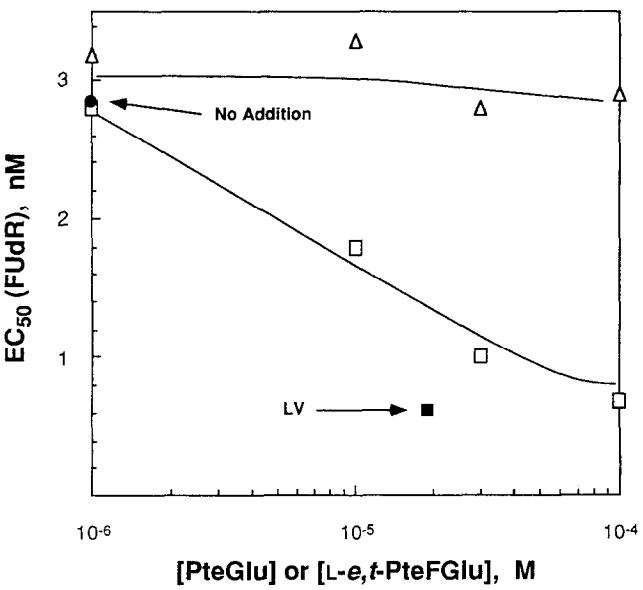


FIG. 5. Potentiation by PteGlu or DL-*e*,*t*-γ-PteFGlu of the potency of 5-FUdR as a growth inhibitor of R30dm cells during continuous exposure. The EC_{50} for inhibition of R30dm growth by 5-FUdR was determined as described in Materials and Methods in the absence of any addition to the medium (●), the presence of 20 μM (6*RS*)-LV (■), or the presence of the indicated concentration of PteGlu (□) or DL-*e*,*t*-γ-PteFGlu (△). The concentration of DL-*e*,*t*-γ-PteFGlu is given as the active L-*e*,*t*-isomers only.

Table 2. Inhibition of growth of CCRF-CEM cells by L-MTX, DL-MTX, L-*t*-γ-FMTX, and DL-*t*-γ- FMTX

Drug	Growth inhibition EC_{50} (nM)	DHFR inhibition* IC_{50} (nM)	TS inhibition K_{is} (μM)	TS inhibition K_{ii} (μM)
L-MTX	14 ± 0	0.72 ± 0.07	58 ± 27	72 ± 12
DL-MTX	30 ± 0	1.18 ± 0.08	ND†	ND
L-*t*-γ-FMTX	102 ± 23	0.84 ± 0.10	ND	ND
DL-*t*-γ-FMTX	133 ± 12	1.35 ± 0.05	50 ± 13	49 ± 6
DL-*e*-γ-FMTX	74‡	1.0‡	42 ± 10	32 ± 7

The drug concentration required to inhibit growth of CCRF-CEM cells by 50% during continuous exposure for 120 hr was determined as described in Materials and Methods. Values are means ± SD for N = 3.

* Data from Hart *et al.* [9].

† ND = not determined.

‡ Data from McGuire *et al.* [8].

these analogs. In continuous exposure, L-*t*-γ-FMTX was 7-fold less potent than MTX as an inhibitor of CCRF-CEM growth (Table 2). At concentrations of L-MTX, DL-MTX, DL-*e*,*t*-γ-FMTX, or L-*t*-γ-FMTX that were equally growth inhibitory, protection by (6*RS*)-LV was afforded with the same concentration-dependence for each drug; at an EC_{95}, 10 nM LV was minimally protective, while 50 nM was fully protective in all cases (data not shown).

DISCUSSION

Initial biological studies focussed on the ability of γ-fluorinated analogs of PteGlu or LV to replace PteGlu or LV as growth factors for folate-depleted H35 cells. The PteGlu analog DL-*e*,*t*-γ-PteFGlu, which is poorly polyglutamylated [9], supported optimal cell growth, although it was 17- to 25-fold less potent than PteGlu itself (Table 1). Since the D-enantiomers are probably inactive [23–25], the potency difference may only be 8- to 12-fold. Two observations concerning metabolism of DL-*e*,*t*-γ-PteFGlu are relevant to interpreting these data. First, the L-enantiomers of DL-*e*,*t*-γ-PteFGlu and its reduced cofactor forms are probably converted to polyglutamates intracellularly, albeit at dramatically reduced rates relative to the protio congeners. This premise is based on data obtained with purified FPGS [9] and our studies [8] showing limited polyglutamylation of the 4-FGlu-containing MTX analog (studies with radiolabeled DL-*e*,*t*-γ-PteFGlu are obviously required to confirm this premise). Second, although DL-*e*,*t*-γ-PteFGlu has not been studied biochemically with respect to every reaction of folate metabolism, its dihydro form is an excellent substrate for CCRF-CEM human dihydrofolate reductase [9]. Since other reactions of folate metabolism also occur at the pterin heterocycle, the enzymes catalyzing these reactions generally utilize analogs of folic acid containing amino acids closely homologous to glutamate quite efficiently (e.g., Refs. 26–28). Since substitution of 4-FGlu for Glu is particularly conservative, it might be expected that folate analogs containing 4-FGlu would be utilized with high efficiency. Thus, if 4-FGlu substitution decreases only polyglutamylation and does not affect other reactions of folate metabolism, our data would indicate that, although polyglutamylation may be essential [3], it is not rate-limiting to growth. (6S)-LV was about 30-fold more potent than PteGlu in promoting growth (Table 1). Presumably, this difference reflects the facts that LV, a reduced folate, is more efficiently transported than is PteGlu [29], and that LV contains a readily utilizable one-carbon unit, whereas PteGlu must first be reduced and a one-carbon unit added before it is useful in folate-dependent reactions. Compared with DL-*e*,*t*-γ-PteFGlu, both γ-FLV species also showed a dramatic increase in growth-promoting potency (Table 1); the γ-FLV were still less potent than LV, however. (6*RS*)-L-*t*-γ-FLV was the least potent, which may reflect the lower FPGS substrate activity of the *threo* diastereomer [9].

DL-β,β-PteF_2Glu was 2- to 3-fold more potent than PteGlu as a growth promoter of folate-depleted H35 cells, when compared on the basis of active L-isomers (Table 1, Fig. 2). Its increased potency may result from more ready transport of L-β,β-PteF_2Glu than PteGlu [11] and/or that its reduced cofactor forms are more efficient than their protio congeners as substrates for folate-dependent enzymes, especially for polyglutamylation [11]. However, since as far as is known F_2Glu-containing species can be converted only to diglutamates [11], these results suggest that the essential functions of folylpolyglutamates can be fulfilled by diglutamates, at least in this model system. Data showing that triglutamates are sufficient for optimal growth has been obtained by Shane and co-workers [30]. These growth promotion data suggest that cofactor forms of β,β-PteF_2Glu are readily utilized in all reactions of folate metabolism; this is an important conclusion since it is a laborious task to test the cofactor forms with each enzyme in folate metabolism.

Above 2 μM, DL-β,β-PteF_2Glu displayed a decreasing ability to support growth of folate-depleted H35 cells, ultimately leading to inhibition of growth below the unsupplemented level (Fig. 2); this effect was not observed with PteGlu. Thus, DL-β,β-PteF_2Glu inhibits its own activity. One explanation for this effect is that the capacity of dihydrofolate reductase is exceeded, and oxidized β,β-PteF_2Glu accumulates. β,β-PteF_2Glu or its diglutamate could either inhibit folate-dependent enzymes directly or, by competition, could decrease polyglutamylation of reduced analogs (e.g. DL-β,β-H_2PteF_2Glu), thereby making them less effective in promoting growth. Metabolite protection studies are consistent with this hypothesis, but do not exclude other explanations.

As in their effects in growth promotion, γ-fluorinated analogs of LV were qualitatively similar to LV in their ability to protect or "rescue" cells from growth inhibition by MTX. However, higher concentrations of (6S)-DL-*e*,*t*-γ-FLV relative to (6S)-LV were required for protection (37-fold) or rescue (58-fold; Fig. 3) than for growth promotion (8-fold). This may reflect the fact that, under the stress of MTX exposure, the poorly polyglutamylatable analog is less

efficiently re-reduced and metabolized to one-carbon adducts and, thus, more preformed γ-FLV is required. The potency of the analog may also be compromised by a further decrease in its already low rate of polyglutamylation as a result of competition for FPGS by MTX and/or the endogenous folylpolyglutamate pool, both of which are present in the folate-replete cells used in these studies.

Growth inhibition of CCRF-CEM cells by continuous exposure to 5-FUdR was potentiated by DL-*e,t*-γ-PteFGlu (Fig. 4) and (6S)-DL-*e,t*-γ-FLV to the same extent seen with PteGlu and LV. As in the studies above, higher concentrations of fluorinated analog relative to its protio congener were required to achieve the same degree of potentiation. Since the polyglutamylation-reduced analogs can potentiate 5-FUdR to the same extent as do natural folates, these data suggest that polyglutamylation is generally not limiting for the potentiation. However, with the polyglutamylation-defective subline R30-dm, 5-FUdR potency was not potentiated by DL-*e,t*-γ-PteFGlu, while PteGlu did potentiate (Fig. 5). This suggests that some level of polyglutamylation is essential for potentiation, since potentiation did not occur when a cell line with very low FPGS activity is treated with a poorly polyglutamylatable analog.

In summary, biological evaluation in a number of test systems of γ-fluorinated folate and LV analogs shows that their properties are qualitatively similar to their protio congeners. These results are mirrored in the effects of a stereochemically pure fluorinated MTX analog, L-*t*-γ-FMTX (Table 2), which are similar to those of MTX. The major difference in each case is quantitative in that higher concentrations of γ-fluorinated analogs are required to achieve the same level of biological effect. Thus, in each case we can conclude that polyglutamylation is not limiting to the effect in cells containing normal FPGS levels. In the case of DL-β,β-PteF_2Glu, the only property assessed thus far is growth promotion. In the physiological concentrations range, DL-β,β-PteF_2Glu was slightly better at promoting growth of folate-depleted cells than was PteGlu. This result indicates that folyldiglutamates are sufficient for active cell proliferation. These two examples show the utility of analogs with altered abilities to undergo polyglutamylation for exploring the requirements for polyglutamylation in cell growth and in biochemical/pharmacological processes.

This work was supported by research grants CA13038, CA43500 and CA16056 (J.J.M.), CA28097 (J.K.C.), and CA25933 (J.G.). The authors are grateful to Robert F. Rotundo for determining the growth promoting activities of these analogs in folate-depleted H35 cells. C. acidi-urici 10-formyl-tetrahydrofolate synthetase was a gift of Dr. J. C. Rabinowitz, University of California, Berkeley.

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