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Effects of D,L-4-fluoroglutamic acid on glutamylation of methotrexate by hepatic cells *in vitro*

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Folypolyglutamate synthetase catalyzes the γ-glutamylation of folates as described in reaction 1.

$$H_4PteGlu_n^* + ATP + L\text{-}Glu \rightarrow H_4PteGlu_{n+1} + ADP + P_i \quad (1)$$

The enzyme is somewhat nonselective with regard to the structure of the folate: numerous folates and antifolates can serve as substrates [1], provided no alterations are made in the glutamate portion of the molecule. The glutamate being added cannot be changed greatly, although some modifications in this structure are possible [1]. An example is replacing the L-Glu by *threo*-FGlu, in which case a single *threo*-FGlu molecule can be added to the folate, resulting in the formation of H_4PteGlu *threo*-FGlu [2]. Further glutamylation of this molecule is prevented by the presence of the fluorine atom adjacent to the γ-carboxyl. Thus, the *threo*-FGlu acts as a terminator of glutamate chain elongation.

The inhibitory properties of FGlu with isolated folylpolyglutamate synthetase necessitated an examination of the effects of this analog on glutamylation in intact cells. For this study we chose to investigate the glutamylation of the antifolate MTX in hepatic parenchymal and H35 hepatoma cells *in vitro*. The advantages of methotrexate are the limited number of glutamylation products and their exact resolution and identification, based upon the earlier characterization of the glutamylation reaction [3–5]. A preliminary account of the results of the current study has been presented [6].

Materials and methods

Male Lewis rats were obtained from Charles River Breeding Laboratories. They were maintained on a 12-hr inverted light–dark schedule and fed Wayne Lab Blox F6 and water *ad lib*. Swims medium S77, folic acid-free Swims medium, Liebowitz L-15 medium, fetal calf serum and horse serum were obtained from Gibco Lab. MTX and [3′,5′,7-^{3}H]MTX were obtained from Lederle Laboratories and Moravek Biochemicals respectively. Both were purified with DEAE cellulose, and the concentrations were determined by ultraviolet spectroscopy [7]. The final specific activity of the radioactive methotrexate was between 5×10^4 and 50×10^4 dpm/nmole. D,L-*erythro,threo*-FGlu and each of the pure *erythro* and *threo* diastereomers were obtained from Calbiochem, and 4-NH_2-10-CH_3PteGlu-γ-FGlu was obtained by chemical synthesis [2].

Hepatocytes were isolated, cultured as monolayers, and incubated with [^{3}H]MTX as previously described [8, 9]. H-11-EC3 cells (referred to as H35 cells) from the Reuber rat hepatoma cells were also cultured as monolayers as described [7]. After 72 hr in culture the cells were changed to folate-free Swims medium S77, for 30 hr, then to fresh medium containing 0.1 μM insulin for 18 hr, at which point a 4-hr incubation with [^{3}H]methotrexate was begun. Incorporation of methotrexate into polyglutamates was measured the same way in hepatocytes and hepatoma cells, and the reaction products containing from two to five glutamate residues were resolved by high-performance liquid chromatography [3, 4]. Standard enzymatic techniques were used for the measurement of ATP and glutamate [10, 11].

The *erythro,threo* mixture of FGlu had no effect on glutamylation in hepatocytes at concentrations up to 5 mM. Glutamylation was reduced in H35 cells with an I_{50} of 0.7 mM when 10 μM MTX and 4 mM glutamine were present in the medium. The *threo* derivative (I_{50} = 0.26 mM) was more effective than the *erythro* derivative (I_{50} = 0.74 mM) of FGlu in inhibiting this reaction. The amounts of all of the individual methotrexate polyglutamate derivatives were reduced by 5 mM *threo* FGlu but the most dramatic decreases were noted with longer-chain-length derivatives (Glu_3, Glu_4 and Glu_5). The presence of 20 mM glutamic acid restored the methotrexate polyglutamate profile of *threo*-FGlu-treated cells to that of the control cells.

We sought to determine if the end product of the reaction of methotrexate and *threo*-FGlu could be detected as has been observed with rat liver folylpolyglutamate synthetase [2]. MTX-FGlu elutes between 4-NH_2-10-CH_3PteGlu$_2$ and Glu_3 on DEAE cellulose [3] and HPLC [4]. None of this material was observed in extracts of FGlu-inhibited cells nor in the medium following incubation. These results suggest that the 4-FGlu was inhibiting polyglutamylation by a mechanism different than that observed with folylpolyglutamate synthetase [3].

* Abbreviations: H_4PteGlu, 5,6,7,8-tetrahydrofolic acid, 5,6,7,8-tetrahydropteroylglutamic acid; methotrexate, MTX, 4-NH_2-10-CH_3PteGlu, 4-amino-10-methylpteroylglutamic acid; L-Glu, L-glutamic acid; FGlu, D,L-4-fluoroglutamic acid (an equimolar mixture of the *erythro* and *threo* diastereomers); methotrexate-γ-FGlu, 4-$NH_2$10-CH_3PteGlu-γ-FGlu, 4-amino-10-methylpteroylglutamyl-γ-D,L-4-fluoroglutamic acid.

The reason for the reduction in methotrexate polyglutamates became apparent when cells containing saturating amounts of MTX polyglutamates were treated with *threo*-FGlu. Under control conditions, a prolonged retention of the polyglutamate pool in H35 cells is observed [3, 12, 13], which is characterized by a more extensive release of shorter-chain-length polyglutamates (Table 1). The polyglutamates lost by the cells have been shown to exit to a very limited extent by leakage of intact polyglutamates and by a more extensive cleavage to MTX which rapidly effluxes from the cell [3, 12, 14]. Greater losses are observed with shorter-chain-length polyglutamates [3].

A much more rapid loss of intact polyglutamates was observed when *threo*-FGlu was added to the medium. The transmembrane passage of these normally impermeable molecules suggests that *threo*-FGlu is impairing the integrity of the cell membrane. Data in support of this come from the observation that *threo*-FGlu caused H35 cells to become permeable to trypan blue, and depleted the cells of ATP and glutamate by 91 and 84% respectively. These data suggest that FGlu is impairing the cellular synthesis and accumulation of methotrexate polyglutamates by acting as an antimetabolite, presumably via one of the glutamate pathways, which results in membrane disintegration.

The effects on cell permeability were observed at millimolar concentrations of FGlu with confluent cultures of H35 cells. Further investigations were conducted to determine the effects of 4-FGlu on H35 cell growth. Much lower concentrations (15–60 μM) exerted inhibition when FGlu was added to the cultures at the time of plating (Fig. 1). *Threo*-FGlu (I_{50} = 17 μM) was more inhibitory than *erythro*-FGlu (I_{50} = 56 μM). Essentially identical inhibition was exhibited against a methotrexate transport-resistant H35 cell subline and against H35 cells grown in a medium lacking folate but supplemented with thymidine and hypoxanthine. The latter results demonstrate that the toxicity of FGlu is not related to folate metabolism in dividing H35 cells. Toxicity was not prevented by a 10-fold excess of glutamate. No effects were observed on the viability of hepatic parenchymal cells. As with confluent cells, 0.1 mM FGlu had no effect on the glutamylation of methotrexate in dividing cells over 4 hr, but 5 mM FGlu caused extensive inhibition.*

Table 1. Effect of D,L-*threo*-FGlu on cellular loss of methotrexate polyglutamates*

	Concentration (nmoles/g)				
		30 hr		30 hr *threo*-FGlu	
Derivative	24 hr Cells	Cells	Medium	Cells	Medium
Glu_1	9.9	4.3	36.0	2.7	44.3
Glu_2	6.3	2.2	3.6	0.5	4.8
Glu_3	45.0	10.6	6.0	9.7	33.1
Glu_4	171.0	126.0	12.0	53.9	97.0
Glu_5	58.0	81.0	2.4	16.9	30.0

* H35 cells were cultured as described in the text and incubated with 10 μM [^{3}H]methotrexate in folate-free Swims S77 medium containing 0.1 μM insulin for 24 hr. The 30-hr data reflect an additional 6-hr incubation without [^{3}H]methotrexate in the presence or absence of 2 mM *threo*-FGlu. The medium after the 24-hr incubation contained only MTX and trace amounts of Glu_2. Methotrexate polyglutamtes were analyzed in the cells by high-performance liquid chromatography [4] and in the medium by DEAE cellulose chromatography [3]. The results are those of a single experiment with duplicate sampling. Two other independent experiments gave essentially identical results.

* J. Galivan, unpublished data.

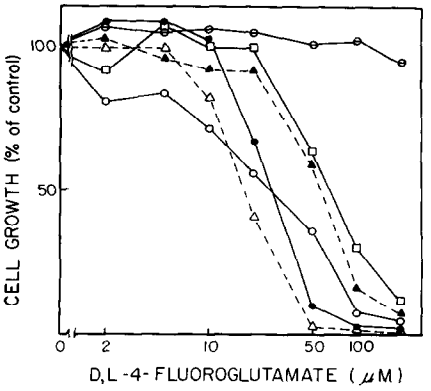

Fig. 1. Inhibitory effects of FGlu on H35 cells and hepatocytes. H35 cells were plated in the presence of the indicated concentration of FGlu. After 72 hr, the number of cells was recorded by trypsinization and the use of a Coulter counter [7]. A mixture of the *erythro* and *threo* derivatives (●) and pure *erythro* (▲) and *threo* (△) were examined. The effect of the mixture was also tested against the transport-dependent, methotrexate-resistant cell line [7, 15] $H35R_{0.3}$ (○) and against H35 cells grown in medium lacking folic acid and supplemented with 50 μM thymidine and 50 μM hypoxanthine (■). *Erythro,threo*-FGlu was also incubated with hepatocytes for 48 hr (⊖), and cells were counted microscopically with the aid of trypan blue exclusion [8]. The results of all experiments are the average of triplicate culture plates.

In conclusion, we have shown that methotrexate polyglutamates in hepatoma cells were reduced by both diastereomers of FGlu, and that the *threo* derivative was more effective than the *erythro* derivative (I_{50}: *threo* = 0.26 mM, *erythro* = 0.74 mM). The effect was prevented by a 10-fold excess of glutamic acid. FGlu appeared to inhibit glutamylation, at least in part, by causing the cells to become permeable and allowing the release of the reaction substrates (ATP and glutamate) as well as the products of the reaction, methotrexate polyglutamates, which are normally retained by H35 cells. This suggests that D,L-4-fluoroglutamate prevents the accumulation of methotrexate polyglutamates by interfering with a glutamate-dependent process in hepatoma cells which is not catalyzed by folylpolyglutamate synthetase. No effect on the glutamylation of methotrexate by hepatic parenchymal cells was observed at 5 mM FGlu.

The effects of *erythro,threo* FGlu and each of the diastereomers on the viability of hepatic parenchymal and H35 hepatoma cells were also examined. At concentrations as high as 5 mM, no effect on cell viability was observed with hepatocytes. FGlu prevented the growth of dividing cultures of H35 cells, and the *threo* derivative (I_{50} = 17 μM) had a slightly greater effect than the *erythro* derivative (I_{50} = 56 μM), whereas confluent cultures were unaffected at these concentrations. The lower concentrations required to prevent growth and lack of reversal by an excess of glutamate suggest that the mechanism of toxicity to growing cells and impairment of glutamylation may be different.

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Selective inhibition of one of the cyclic AMP phosphodiesterases from rat brain by the neurotropic compound rolipram

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Among the novel neurotropic drugs which have been described in the past few years, the cyclic nucleotide phosphodiesterase inhibitor rolipram (4-[3-cyclopentyloxy-4-methoxyphenyl]-2 pyrrolidone) proves to be of major interest, as it induces in animals a peculiar profile of behavioral modifications [1, 2] and exhibits antidepressant potentialities, in animal trials [3, 4] as well as in preliminary clinical observations [3]. Interestingly, it induces specific increases in cyclic AMP level without affecting cyclic GMP level, in brain cortical slices [5]. Large increases in cyclic AMP level are also observable in several areas of the brain, after *in vivo* treatment of rats by rolipram [6]. *In vitro*, in crude preparation, it proves to be a rather potent inhibitor (in the micromolar range) of cyclic AMP-hydrolyzing, Ca^{2+}-independent phosphodiesterase of rat brain, while it weakly affects cyclic GMP-hydrolyzing phosphodiesterase [5]. In other tissues, such as in beef aorta [7], or in rat heart (our laboratory, unpublished results), it inhibits with a high selectivity the partially purified cyclic AMP-specific isoenzyme. Recently, Davis described the preferential effect of rolipram on the Ca^{2+}-independent fraction of rat brain cortex phosphodiesterase, separated by gel filtration and further cleared up of Ca^{2+}-dependent enzyme by passage on a calmodulin affinity column [8]. Or, gel filtration resolves only two peaks of activity from cerebral tissue [9–11], while other methods point out up to six forms of phosphodiesterase [11–15]. The Ca^{2+}-insensitive brain fraction might thus be constituted of several components with various sensitivities to rolipram. The isolation of an enzyme species particularly sensitive to rolipram inhibition would point it out as a possible candidate as the pharmacological target of the drug.

Materials and methods

Rat brains minus cerebella were homogenized in 9 vol. of 5 mM Tris–HCl pH 7.5, 0.32 M sucrose, in a Teflon–glass potter, and centrifuged for 1 hr at 105,000 *g*. Freshly prepared supernatant was submitted to isoelectric focusing procedure on Sephadex flat gel bed, as in [16]. Alternatively (membrane-bound enzyme studies), the tissue was homogenized in 9 vol. of 20 mM Tris–HCl pH 7.5, 1 mM $MgCl_2$, 0.1 mM dithiothreitol. A first 1000 *g* pellet was discarded. The supernatant was centrifuged for 1 hr at 105,000 *g*. The resulting pellet was washed one time and resuspended in the initial volume of the same buffer containing 1% non-ionic detergent Lubrol PX. The medium was submitted to sonication for 1 min (Branson Sonifier, 80 pulses, power 4), and centrifuged for 1 hr at 105,000 *g*. Nearly 80% of phosphodiesterase activity was found in the supernatant. This solubilized fraction was submitted to isoelectric focusing as in [16].

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Phosphodiesterase was assayed by the radioisotopic procedure of Thompson and Appleman [9], modified as described in [17]. Assay medium contained 100 mM Tris–HCl pH 8, 5 mM $Mg Cl_2$, 0.5 mg/ml bovine serum albumin, 0.25 μM cyclic nucleotide.

Results and discussion

As isoelectric focusing on granular gel plate proves an efficient technique for phosphodiesterase fractionation [16], 105,000 *g* supernatant of rat brain was submitted to this procedure in a 4–8 pH range. A reproducible pattern of phosphodiesterase activity was observed (Fig. 1 A,B). A group of peaks ranging from pI 4.9 to 5.4 hydrolyzed preferentially cyclic GMP. Two individualized cyclic AMP-hydrolyzing peaks focused at pI 5.6 and 6. The cyclic