ORIGINAL ARTICLE

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Glutamyl hydrolase and the multitargeted antifolate LY231514

Abstract *Purpose*: To examine the activity of glutamyl hydrolase (GH) on the poly-γ-glutamates of multitargeted antifolate (MTA) (LY231514) and the effect of enhanced GH on the pharmacological activity of MTA. *Methods*: Expressed and purified GH were used to study the enzymatic cleavage of MTA poly-γ-glutamates and wild-type and GH-enhanced H35 hepatoma cell lines to evaluate growth inhibition. *Results*: MTA tri- and penta-γ-glutamates were good substrates for human GH, having higher rates than MTX tri- and penta-γ-glutamates. Preferential hydrolysis with human enzyme occurred at the two γ-glutamyl bonds at the carboxyl end of the molecule, whereas the rat enzyme preferred the innermost γ-linkage. Incubation of rat H35 hepatoma cell lines with MTA resulted in the intracellular accumulation of primarily tetra-, penta-, and hexa-γ-glutamate. The formation of these were markedly reduced in H35D cells, which is a line resistant to antifolates chiefly through enhanced cellular levels of GH activity. *Conclusions*: MTA poly-γ-glutamates are effective substrates for GH and their pharmacological effectiveness bears an inverse relationship to cellular GH activity. This observation, along with enhanced resistance to MTA of thymidylate synthase-amplified cells, substantiates the importance of the poly-γ-glutamates of MTA inhibiting TS as the primary target. Further evidence for the inverse relationship of GH to classical antifolate pharmacological activity is established.

Key words Glutamyl hydrolase · Folylpoly-γ-glutamate hydrolase · MTA · LY231514 Poly-γ-glutamates · Antifolates

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Introduction

Antifolates have been utilized effectively against neoplastic diseases during the latter half of the twentieth century [1, 13]. There has been considerable success against many cancers, especially those of the blood, but effectiveness against solid tumors has been more elusive. Methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR), has been the most widely used of these agents, and also has been most instructive about the pharmacology of the antifolates. It has been established as a model compound to study transport systems, polyglutamylation, drug-target interactions, acquired drug resistance, and rescue protocols [1, 13].

Approaches to broaden the activity of the antifolates and heighten their specificity have included the synthesis of modified folate analogues that have targets other than DHFR. Several have been designed that inhibit thymidylate synthase (TS) and glycine aminoribotide formyltransferase (GARFT) [1, 6, 13, 20, 21]. A recent development is the synthesis of MTA (multitargeted antifolate, LY231514, *N*-[4-[2-(2-amino-3,4dihydro-4-oxo-7Hpyrrolo[2,3-d]pyrimidin-5-yl)-ethyl]-benzoyl]-L-glutamic acid). MTA as its poly-γ-glutamate derivatives inhibits several folate-utilizing enzymes, although the primary target appears to be TS [6, 20, 21]. Other enzymes that may be impaired by MTA polyglutamates and contribute to its cytotoxicity are DHFR and GARFT [20, 21].

Since the cellular retention and enzyme binding of MTA are enhanced by poly-γ-glutamylation, its conversion to these derivatives is an important component of its activity [21]. MTA has already been shown to be one of the best substrates for folylpolyglutamate synthetase (FPGS) when compared to numerous other classical antifolates [22]. The only other compounds that share its high affinity and Vmax for the enzyme are *N*-(5-[*N*-(3,4-dihydro-2-methyl-4-oxoguinazoline-6-ylmethyl)-*N*-methylamino]-2-thenoyl)-L-glutamic acid (ZD1694, Tomudex) and 5,10-dideazatetrahydrofolate (DDAT HF) [6].

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As yet no studies have been performed regarding the cleavage of MTA poly-γ-glutamate derivatives by γ-glutamyl hydrolase (folylpolyglutamate hydrolase, GH, with hGH referring to human GH and rGH referring to rat GH). The activity of this enzyme has been shown to be an important determinant of intrinsic and acquired resistance to antifolates [9, 12, 15]. New information on GH has made it more amenable to study with regard to its preclinical pharmacology and its role in the clinical effectiveness of antifolates. These studies include elucidation of cDNAs, protein sequence, and production of antibodies to the rodent and human enzymes [5, 16, 27, 28], and the chromosomal location of the human gene and its tissue-specific expression [25].

Materials and methods

MTAGlu$_5$[1], MTAGlu$_3$, MTA and [H^3]MTA were provided by Lilly Research Laboratories, Eli Lilly company (Indianapolis, Ind.). MTXGlu$_5$, Folate(Fol)Glu$_5$ and p-aminobenzoyl(pABA)Glu$_5$ were purchased from Dr. B. Schirck's Laboratories (Jona, Switzerland). Acetonitrile (HPLC grade) was purchased from J.T. Baker (Phillipsburgh, N.J.) and all other chemicals from Sigma Chemicals (St. Louis, Mo). Baculovirus-expressed hGH purified on a Protein-Pak CM column [16], and purified rat rGH from H35 cell conditioned medium [27] were used for the experiments.

Growth inhibition study

Cells were plated at 1×10^4 cells in 200 μl Swim's medium containing 10% FBS per well in a 96-well plate (Corning Glass Works, Corning, N.Y.). After 3 h, MTA was added to the cultures. Outgrowth was measured after 72 h as previously described [15].

Synthesis of MTA polyglutamates by H35, H35FF and H35D cells

Cells were grown in Swim's medium containing 20% horse serum and 5% fetal bovine serum (FBS) for 72 h and then the medium was replaced with Swim's medium containing 10% FBS for the next 24 h. At 96 h cells were incubated with 0.5 μM [^{3}H]MTA (specific activity, 8.2×10^5 dpm/nmol) for 2, 4, 6, or 24 h. [^{3}H]MTA polyglutamates in cell extracts were separated by HPLC with a linear gradient of 2 to 12% acetonitrile in 0.1 M sodium acetate (pH 5.5) over 20 min and traced at 280 nM. Each MTA poly-γ-glutamate peak was identified using MTA, MTAGlu$_3$ and MTAGlu$_5$ as standards. The radioactivity of each fraction was counted by liquid scintillation (LS-3801, Beckman) and is expressed as picomoles per milligram cell protein.

GH assay

Substrates were incubated with hGH or rGH at 37 °C for 10 min. The reaction mixtures were analyzed by HPLC/UV for heterocycle-containing glutamate products [16, 24] and by HPLC/fluorescence

detection for the released γ-glu$_n$ products after derivatization with orthophthaldehyde (OPA) [16]. For the kinetic studies, the rate was measured as the disappearance of the substrate.

Results

The relative activity of hGH on the poly-γ-glutamates of MTX, MTA, folate, and p-aminobenzoate (PABA) was evaluated (Fig. 1). With the pentaglutamate derivatives of each substrate, MTAGlu$_5$ had the highest rate of cleavage, being about 30% greater than MTXGlu$_5$. The triglutamate of MTA was essentially equivalent to MTXGlu$_5$. PteGlu$_5$ and PABAGlu$_5$ were hydrolyzed more slowly at concentrations between 100 and 200 μM.

The substrate and product profile during the course of the reaction with the pentaglutamate substrates of MTA, MTX, and folate were examined (Fig. 2). The amount of substrate remaining is inversely related to the velocity of the reaction. As shown in Figs. 1 and 2, the relative velocity was MTAGlu$_5$ > MTXGlu$_5$ > FolateGlu$_5$. The product profile showed that all possible glutamyl derivatives of folates and the antifolates were present during the course of the reaction. An interesting feature with hGH is that the product with two fewer glutamates exceeded that with one glutamate removed. With MTX poly-γ-glutamates this result is related to the fact that the enzyme can cleave the ultimate and pen-
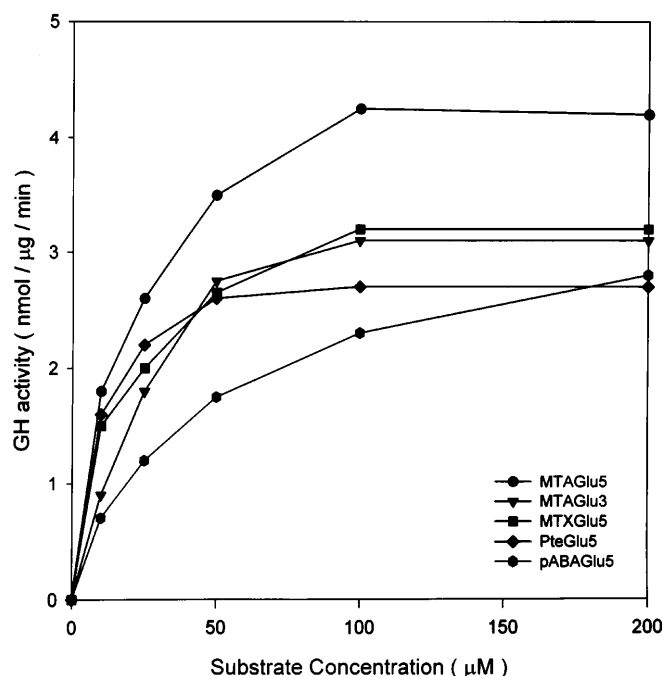

Fig. 1 Concentration dependence of MTAGlu$_5$, MTAGlu$_3$, MTXGlu$_5$, FolGlu$_5$ and pABAGlu$_5$ as substrates for hGH. The substrates were incubated with hGH at 37 °C for 5 to 30 min. The substrates and hydrolyzed products were analyzed by HPLC/UV [4]. GH activity was calculated by the disappearance of the substrate. The maximum reaction rate (nmol/μg/min) at each substrate concentration was plotted against the substrate concentration. Each point represents the average of two experiments

[1] In all cases Glu$_n$ indicates the total number of glutamate residues in the poly-γ-glutamate derivatives. The parent molecules that contain one glutamate residue are indicated with reference to the glutamate such as MTA and MTX. Thus the MTAGlu$_5$, MTXGlu$_5$, or FolGlu$_5$ contain the glutamate present in MTA, MTX, or Fol plus four additional γ-linked glutamates.
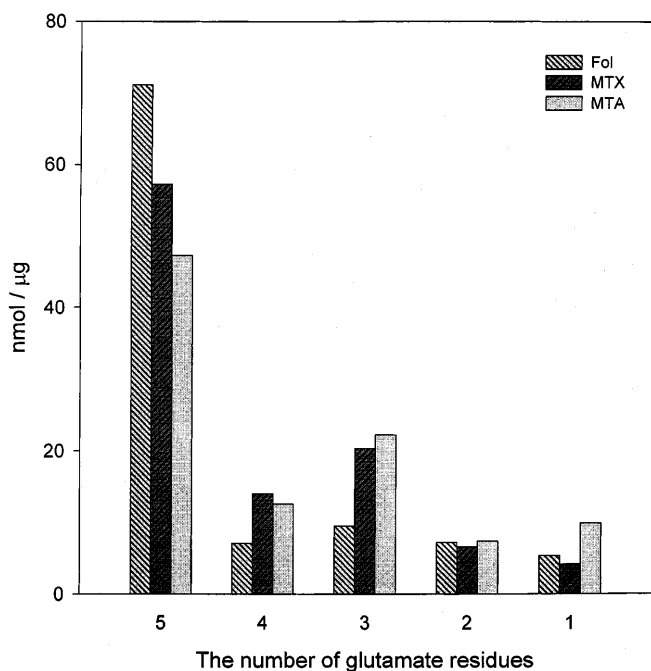
Fig. 2 Distribution of substrate and hydrolyzed products of the pentaglutamates of MTA, MTX and folate catalyzed by hGH. Substrates (100 μM) were incubated with hGH at 37 °C for 10 min. Hydrolyzed products of pterin-containing γ-glutamates were analyzed by HPLC/UV [4]. Data represent the average of two or three experiments

Table 1 Formation of γ-glutamate products from the hydrolysis of different substrates by hGH. Substrates (100 μM) were incubated with hGH at 37 °C for 15 min. γ-Glu$_n$ species in the reactants were analyzed following OPA-derivatization by HPLC with a fluorescence detection system [16]. γ-Glu$_3$, γ-Glu$_4$, and γ-Glu$_5$ were not detected. Values are the average of two experiments

Substrate	γ-Glu$_2$	Glu
	(nmol/μg)	
MTAGlu$_5$	27.2	37.6
MTXGlu$_5$	22.1	27.1
FolGlu$_5$	16.7	24.9

ultimate γ-linkages [16]. The same appeared to be the case with MTA and folate polyglutamates. An analysis of the released glutamate species verified this conclusion since only di-γ-Glu and Glu were released (Table 1). The cleavage of PABAGlu$_5$ followed a similar pattern (data not shown). In several hundred assays using various pteroyl or PABA pentaglutamates as substrate in this study and an earlier one [16] we have never observed the release of γ-glu$_3$ or γ-glu$_4$. This does not absolutely rule out the possibility that hGH can cleave interior γ-linkage, since small amounts may form and be further hydrolyzed to Glu. If such reactions occur our data suggest that they are minor. Current studies using mutagenesis and molecular modeling are underway and should unambiguously establish the absence of hydrolysis at sites internal to the outer two γ-linkages.

An interesting substrate that has relatively high activity with hGH and can rapidly determine whether hGH is cleaving both outer γ-linkages is the triglutamate of MTA. When MTAGlu$_3$ hydrolysis is measured as a function of time, the primary product released is MTA with lesser amounts of MTAGlu$_2$ (Fig. 3). Measurement of the release of the glutamate species from MTA Glu$_3$ reveals that γ-Glu$_2$ exceeds that of Glu in the initial phases of the reaction. If the reaction is allowed to continue indefinitely or more enzyme is added the ultimate products of the reaction are MTA and Glu (data not shown).

MTAGlu$_5$ was tested as a substrate for rGH in order to determine whether its hydrolysis is the same as the pentaglutamates of folate and MTX [24, 27]. The data in Table 2 show that MTAGlu$_5$ is cleaved with only MTA
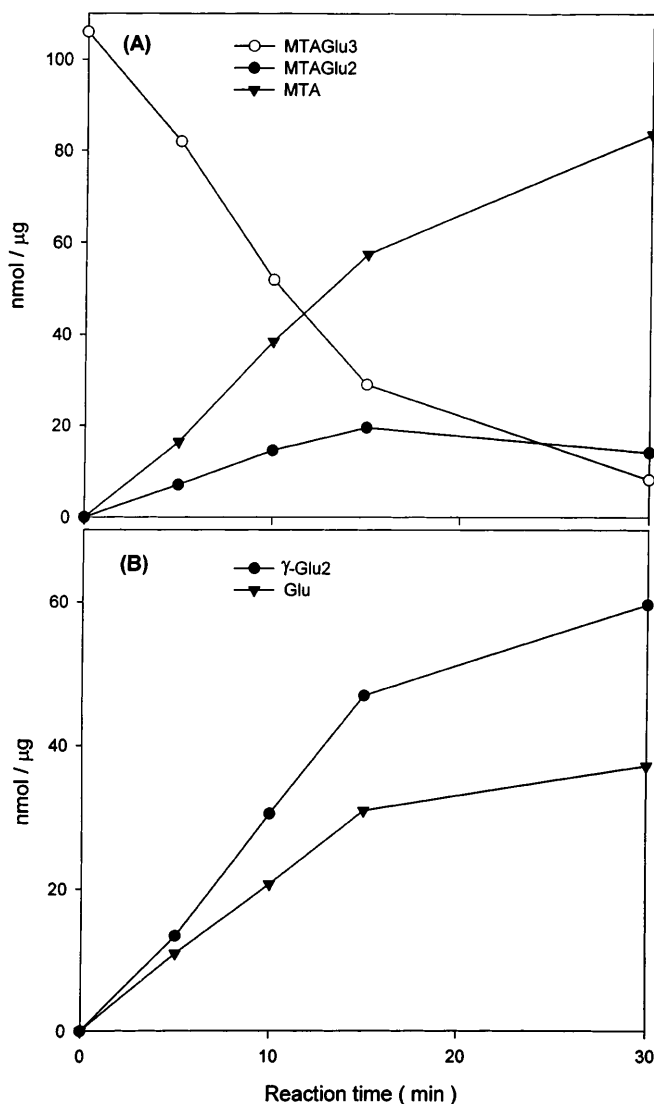

Fig. 3A, B Time-dependence of hydrolyzed products of MTAGlu$_3$ by hGH. MTAGlu$_3$ (100 μM) was incubated with hGH at 37 °C for the indicated times. Reactants were analyzed for (**A**) MTA-containing products by HPLC/UV and (**B**) released γ-Glu$_n$ species

Table 2 Hydrolysis of pentaglutamates of MTA, MTX and folate by the purified rGH of conditioned medium of H35 cells. Substrates (100 nmol) were incubated with the purified rGH of conditioned medium of H35 rat hepatoma cells at 37 °C for 5 min. The reactants were analyzed by HPLC/UV (x MTA, MTX or folate depending upon the experiment)

Substrate	Substrate remained	Hydrolyzed products	
	x-Glu$_5$	x-Glu$_{4,3\&2}$	x
	(nmol)		
MTAGlu$_5$	73.2	0	26.7
MTXGlu$_5$	74.3	0	25.6
FolGlu$_5$	70.7	0	29.3

appearing and no intermediate MTAGlu$_n$ derivatives. This is consistent with the cleavage of MTX and folate pentaglutamates, with the innermost γ-linkage being the preferential site of hydrolysis by the rat enzyme. As with the human enzyme the ultimate products of the reaction are MTA and Glu.

In attempting to understand the cellular pharmacology of MTA and its polyglutamate derivatives the rodent hepatoma H35 cell line was utilized. This line is particularly interesting because it is the only one available that has a highly elevated level of rGH as a result of acquired drug resistance. The subline is 60-fold resistant as a result of a stepwise increase in the concentration of DDATHF and is also resistant to MTX and 10-propargyl-5,10-dideazafolic acid [15]. The data in Table 3 demonstrate its resistance to MTX and MTA, to which it is 70- and 55-fold resistant, respectively. A subline resistant to TS inhibitors by virtue of TS amplifications, H35FF, was also at least 2500-fold resistant to MTA.

The polyglutamate profiles of MTA were examined in H35, H35D (GH-increased), and H35FF (TS-amplified) cells. The results are demonstrated in Fig. 4. The concentration of MTA (0.5 μM) is approximately 80% inhibitory to wild-type cell growth and has no effect on the growth of H35D and H35FF sublines. Over the first 4 h the tetra- and penta-γ-glutamates of MTA predominated with little di- and triglutamate in the parent cell line. The MTA poly-γ-glutamate profile in H35FF was similar to that in the parent cell line. Over 4 h the H35D cells had less than 10% MTA poly-γ-glutamate compared to that found in control cultures.

Table 3 Growth inhibition of H35, H35FF and H35D cells by MTX and MTA. Cells were incubated in the presence of drug for 72 h and the growth was measured as described in Materials and methods. (IC$_{50}$ the concentration causing 50% inhibition of growth of untreated cells, H35 wild-type, H35D resistant subline with increased GH [15], H35FF TS-amplified subline [14]). Values are means ± SD (n = 5)

Cells	MTA	MTX
	IC$_{50}$ (μM)	
H35	0.08 ± 0.01	0.01 ± 0.002
H35D	4.4 ± 0.7	0.7 ± 0.1
H35FF	>250	0.014 ± 0.003

More accumulated over 24 h, predominantly as the pentaglutamate derivative. However, the total of longer chain length poly-γ-glutamates of MTA (Glu$_4$, Glu$_5$, and Glu$_6$) was severely reduced compared to the parent cell line.

Discussion

MTA, or LY231514, is a folate analog that was developed by structure-activity relationship studies of the antipurine antifolate DDATHF (23). Clinical activity has been shown against a number of human tumors including colorectal, breast, pancreatic and non-small-cell lung [2, 3, 7, 10, 11, 17, 18, 19, 22]. MTA can inhibit a number of folate-utilizing enzymes. These are TS, GARFT, and DHFR. The first of these enzymes is probably the primary target of MTA, against which it is active as poly-γ-glutamate derivatives [20, 21].

MTA is a highly efficient substrate for FPGS forming primarily the tetra- and penta-γ-glutamate products when incubated with the enzyme at a concentration of 1 μM [21]. This profile is similar to that seen when radiolabelled MTA is incubated with rat hepatoma cells. The major difference in intact cells is the appearance of significant amounts of the hexa-γ-glutamate. The latter observation is not surprising since poly-γ-glutamate profile of antifolates in intact cells contains longer chain length species than parallel experiments with the isolated enzyme [8]. When the MTA poly-γ-glutamate profiles in intact cells (Fig. 4) are compared with the properties of isolated FPGS [20, 21] it appears as though the enzymatic characteristics of FPGS are a major determinant of the chain length of intracellular MTA polyglutamates.

MTA poly-γ-glutamates are efficient substrates for GH. The rate of hydrolysis of MTAGlu$_5$ exceeds that of MTXGlu$_5$ with hGH and is equivalent to that with rGH. The preferred sites of hydrolysis with hGH and rGH are identical to those observed with other analogs of folic acid (Fig. 5, data shown in Fig. 2, Tables 1 and 2). The ultimate products, given sufficient reaction time or GH concentration, are MTA and Glu with both rat and human enzyme.

Studies with drug-resistant cell lines confirm and extend earlier mechanistic evaluations of MTA [3, 4]. The fact that TS-amplified H35 cells are more than 2500-fold resistant to MTA confirms this enzyme as the primary target for MTA. H35D cells, which have acquired resistance to DDATHF chiefly through enhancement in rGH, are 55-fold resistant to MTA. These results demonstrate the importance of the poly-γ-glutamate derivatives of MTA in being an effective inhibitor of TS and cell growth.

The quantitative reduction in poly-γ-glutamates in response to enhanced GH in H35D cells is shown in Fig. 4. During the first 6 h of incubation with radiolabelled MTA there is more than a 90% reduction in poly-γ-glutamate formation, which is somewhat less (83%)

Fig. 4 MTA polyglutamates in H35, H35FF and H35D cells. Cells were grown for 96 h in Swim's medium containing 20% horse serum and 5% FBS and then incubated with 0.5 μM [^{3}H]MTA in Swim's medium containing 10% FBS for 2, 4, 6 and 24 h. MTA polyglutamates in cell extracts were analyzed as described in Methods. The results are the average of duplicate experiments
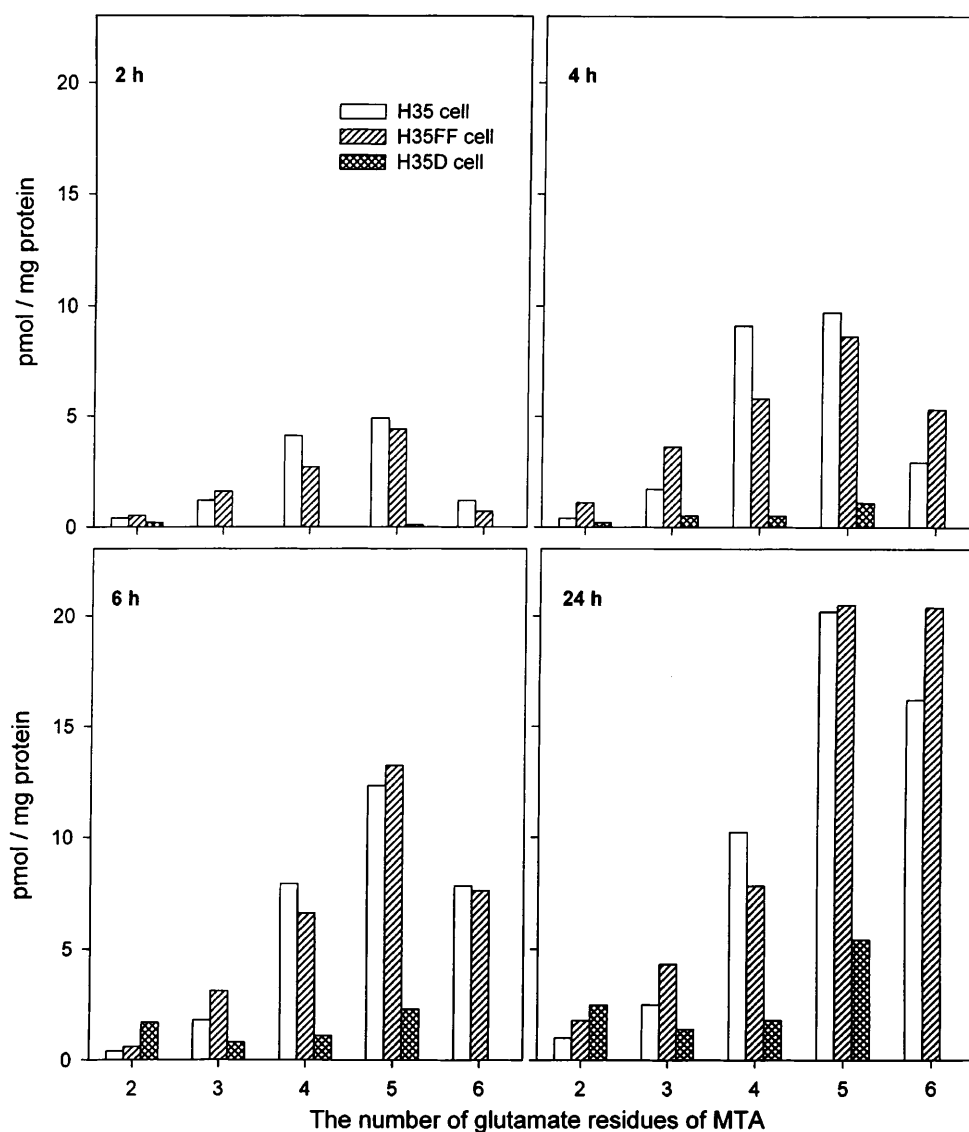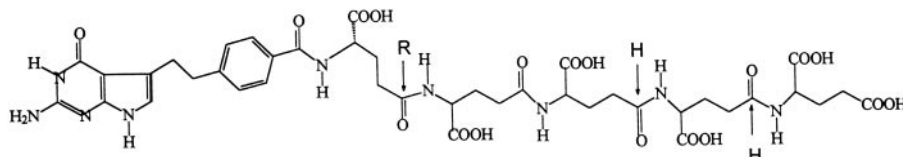
Fig. 5 Structure of MTA pentaglutamate and primary cleavage sites for hGH and rGH. H↓ and R↓ indicate the favored hydrolytic sites for hGH and rGH, respectively

after 24 h. There might be concern that the enhanced rGH in the cells would reduce the intracellular folates and cause greater sensitivity to antifolates such as MTA. This appears not to occur because the effects of elevated GH on the folate pools are much less dramatic than on the antifolates [15, 26]. These results provide further evidence for the inverse relationship between GH levels and the pharmacological effectivenss of classical antifolates.

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