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γ-Glutamyl Hydrolase from Human Sarcoma HT-1080 Cells: Characterization and Inhibition by Glutamine Antagonists

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SUMMARY

Elevated γ-glutamyl hydrolase (GGH) activity as a contributing factor in mechanisms of acquired and intrinsic antifolate resistance has been reported for several cultured cell lines. Despite this, little is known about this enzyme, especially the human species. Using the human HT-1080 sarcoma line, we observed the secretion of GGH activity into media during culture (a phenomenon that could be markedly stimulated by exposure to NH_4Cl) and an acidic pH optimum for *in vitro* catalytic activity of the enzyme. These properties are consistent with a lysosomal location for the enzyme. Unlike rodent GGH, preparations of HT-1080 enzyme (purified ≤2000-fold) displayed exopeptidase activity in cleaving successive end-terminal γ-glutamyl groups from poly-L-γ-glutamyl derivatives of folate, methotrexate (MTX), and *para*-aminobenzoic acid substrates and a marked preference for long-chain polyglutamates (K_m values for glu_4 versus glu_1 derivatives were 17- and 15-fold lower for folate and MTX versions, respectively). Using an *in vitro* assay screen, several glutamine antagonists [i.e., 6-diazo-5-oxo-norleucine (DON), acivicin, and azaserine] were identified as human GGH inhibitors, with DON being the most potent and displaying time-dependent inhibition. In cell culture experiments, simultaneous exposure of DON (10 μM) and [^{3}H]MTX for 24 hr resulted in modest elevations of the long-chain γ-glutamyl derivatives of the antifolate for HT-1080 and another human sarcoma line. These compounds may serve as useful lead compounds in the development of specific GGH inhibitors for use in examining the relationship between GGH activity and antifolate action and may potentially be used in clinical combination with antifolates that require polyglutamylation for effective cellular retention.

Over the past decade, the importance of the role of polyglutamylation in the cellular retention of both folic acid derivatives and antifolates has become more widely recognized. As polyglutamyl derivatives, these metabolites are usually the preferred substrates for folate-dependent enzymes, are far less permeable to the plasma membrane, and may also determine subcellular localization (1–3). Prolonged retention of one antifolate compound commonly used as an antineoplastic agent, MTX ($4\text{-}NH_2\text{-}10\text{-}CH_3\text{-}Pte_{glu1}$), results in more effective inhibition of dihydrofolate reductase and is thought to provide the basis for tumor selectivity (1). The polyglutamylation process is dependent on at least two cellular enzyme activities: folylpolyglutamate synthetase (EC 6.3.2.17) which adds successive glutamate groups to the γ-glutamyl side chain of folates/antifolates, and GGH (EC 3.4.22.12), which removes (hydrolyzes) the polyglutamate side chain via either an endopeptidase or exopeptidase activity. In many cases, the second enzyme is sequestered into lysosomal bodies, and the rate of transport of folylpolyglutamate into and out of this organelle may also regulate the degree of polyglutamylation (4).

A considerable amount of information on folylpolyglutamate synthetase has been elucidated, including its catalytic properties (2, 5), and after isolation and sequencing of the human cDNA (6), work has progressed to recombinantly express the enzyme and determine features of transcriptional control (7). By contrast, relatively little is known about GGH, although the cDNA sequence for the rat enzyme was recently reported (8), and some information on the properties of this enzyme from various species is known. At the enzymological level, available information and its relevance to human GGH are confused by the fact that enzyme content and specificities (endopeptidase or exopeptidase activity) not only vary among species but also cell and tissue type (2, 9–11). It seems that this group of enzymes falls into one of two classes; one that is lysosomal in origin (10, 12–15), with an acid pH optimum, and another, smaller group that has a neutral or alkaline pH optimum. It is the enzyme from the former group that seems to be of importance in regulating folate and antifolate polyglutamate chain lengths (1, 2, 16, 17). Of this class, the bovine hepatic enzyme and the human intracellular jejunal

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ABBREVIATIONS: GGH, γ-glutamyl hydrolase; Pte, pteroyl; HPLC, high performance liquid chromatography; MTX, methotrexate; pAB, *para*-aminobenzoyl; DTT, dithiothreitol; DON, 6-diazo-5-oxo-L-norleucine; glu_n, γ-linkage of successive L-glutamyl residues, where *n* is number of residues.

enzyme have been studied most extensively. Bovine enzyme was purified to (apparent) homogeneity, confirmed to be a glycoprotein with highly reactive sulfydryl groups, and displayed an apparent molecular mass of 108 kDa (12). Although the partially pure human intracellular jejunal enzyme displayed a much lower apparent molecular mass on gel filtration (75 kDa), many of its other properties were similar to those of the bovine enzyme, including the ability to cleave both terminal and internal linkages of folylpolyglutamates (10). This activity preference differs from the strict exopeptidase action found for GGH from human jejunal brush border cells, which probably plays a role in the digestion of dietary folate (14), and very early work with human liver (lysosomal) GGH (18).

Other studies from the laboratories of Galivan and Sirotnak have focused on rodent forms of GGH and, in particular, the secreted forms harvested from cultured cell lines (11, 19). Interestingly, the enzymological properties of the secreted GGH are not different from the cellular (lysosomal) form. Purified GGH secreted from rat H35 hepatoma cells was shown to have extensive carbohydrate modification, and the deduced amino acid sequence from the cDNA sequence indicates a molecular mass of 33.4 kDa for the peptide component of this enzyme species (8).

Clearly more information on the human enzyme or enzymes is necessary to better define the role of GGH in the regulation of intracellular polyglutamate levels (and therefore cellular retention) of folates and antifolates in human cells. Here, we report the partial purification and characterization of GGH from the human fibrosarcoma cell line HT-1080. Purified GGH preparations were also used to test a broad range of potential inhibitor compounds and certain glutamine antagonists demonstrated inhibitory activity. One of these agents, DON, was found to enhance the cellular retention and cytotoxicity of MTX.

Experimental Procedures

Materials. Polyglutamylated derivatives of pteroyl (Pte), 4-NH_2-10-CH_3-Pte, and pAB poly-γ-L-glutamates were purchased from Dr. B. Schirks Laboratories (Jona, Switzerland). The only exception to this was MTX, which was from Lederle Laboratories (Pearle River, NY). [^{3}H]MTX (20 Ci/mmol) was purchased from Moravek Biochemicals (Brea, CA). Reagents for enzymatic assays of β-hexosaminidase and dihydrofolate reductase were from Sigma Chemical (St. Louis, MO) or as described (20). Affinity media Reactive Green 5, organomercurial agarose, gel filtration molecular mass standards, and reagents for cell cytotoxicity measurement were also from Sigma. Sephacryl S-200 was from Pharmacia (Piscataway, NJ). Several compounds used in the inhibition screen were kindly donated: 2-mercaptomethylglutaric acid was from Dr. T. I. Kalman (State University of New York, Buffalo, NY), ICI 198583-γ-D-glu was from Dr. A. L. Jackman (Institute of Cancer Research, Surrey, UK). All other chemicals were of the highest purity available.

Cell culture and preparation of extracts. Various cancer cell lines, including the soft tissue sarcoma line HT-1080, were obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI-1640 containing 2 mM glutamine, 10% fetal bovine serum, and antibiotics. The cytotoxic effect of various agents on the HT-1080 line, using variable concentrations and exposure times, was measured by cell counting or the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide assay (21).

Extracts of HT-1080 cells for enzyme purification were prepared from mid-log growth cultures. After trypsinization and extensive washing with phosphate-buffered saline, cell extract was prepared by sonification and centrifugation steps as previously described (20).

Activity assays. GGH activity was measured at 37°, pH 4.5, using 50 μM 4-NH_2-10-CH_3-Pte_{glu5} as substrate (unless otherwise stated) and standard assay buffer consisting of the MTEN buffer system (22) with 1 mM $ZnCl_2$ and 2 mM DTT. Portions of the assay mix were sampled at various time points, placed in a boiling water bath for 5 min, and then centrifuged at 14,000 × *g*. Supernatants were used directly in HPLC analysis to resolve and quantify products as previously described (11). During the course of this work, a capillary electrophoresis procedure was developed for use in quantifying substrate and reaction products of GGH assays (23) and used primarily in inhibitor screening experiments. GGH activity was calculated from the percentage of substrate degraded per unit time (23). One unit is defined as the amount required to convert 1 μmol of substrate/min. Specific activity values (units/mg) were calculated from assay time points where < 20% of substrate was consumed.

β-Hexosaminidase activity was measured at pH 4.6 using the substrate *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminidine as previously described (4, 24). The dihydrofolate reductase and total protein assay protocols have been previously described (20).

Secretion of GGH in cultured HT-1080. Seeded HT-1080 cells were grown to early log phase; then, a portion of medium was taken and snap-frozen in liquid N_2. The remaining culture was spiked with 10 mM NH_4Cl, and additional samples of media were taken at days 1, 2, and 5. Medium for a duplicate flask of cells (without added NH_4Cl) was also sampled at these times. Total protein and enzymatic activities for GGH, β-hexosaminidase, and dihydrofolate reductase were measured in all samples. The background activities contributed from media alone (close to negligible) was subtracted from all measurements.

Purification of GGH from HT-1080. Three to 4 ml of clarified crude HT-1080 lysate was dialyzed against buffer A (25 mM Tris·HCl, pH 7.5) and then applied to a 6-ml column of Reactive Green 5 gel previously equilibrated with identical buffer. Throughout the run, flow rate was maintained at 0.8 ml/min, eluate A_{280} was continuously monitored, and 3-ml fraction volumes were collected. NaCl solutions prepared in buffer A were used to desorb enzyme, and approaches were used in different runs: either (a) a single linear gradient of 0–1.5 M NaCl (100 ml total volume) or (b) multiple step gradients and washing phases. In the second approach, two linear gradients were used (40 ml total volume each) with an intervening wash step of salt strength equivalent to the maximum achieved in the first gradient.

Fractions containing activity from the previous step were pooled, concentrated on YM30 membranes (30 units of Centriprep; Amicon, Beverly, MA), and then dialyzed against buffer B (50 mM Tris·HCl, pH 7.2). Material was then applied (at a flow rate of 0.22 ml/min) to a 1.5-ml gel-bed volume column of organomercurial agarose previously equilibrated with buffer B. After extensive washing (~30 ml), GGH was described with buffer B containing 10 mM DTT. GGH active fractions were pooled and concentrated as before.

Gel filtration on Sephacryl S-200 was performed using various preparations of partially pure GGH in the presence or absence of thiols. Optimal results for further purification of organomercurial agarose-purified material was obtained by direct application of active fractions without prior dialysis. In these instances, a column of 1.5 cm (i.d.) × 60 cm (height) was used and running buffer consisted of 20 mM Tris·HCl, pH 7.0, 10 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM DTT.

Characterization of GGH. Properties of GGH were investigated using preparations of purity of >3 units/mg (i.e., purified ≥1500-fold from crude extract); this included experiments examining the effect of anions, detergents, and urea on GGH activity. The S-200 column and conditions used for estimation of native molecular mass of GGH was the same as that for purification as described above and in the figure legend. Isoelectric focusing was performed using a Hoefer gel

unit (GT 1) and operated, essentially, under conditions recommended by the manufacturer (Hoefer Scientific Instruments, San Francisco, CA).

Substrate kinetic analysis. Enzyme was incubated in standard GGH assay buffer (37°) with the different substrates at concentrations (final) of 0.2–250 μM. Incubation times varied from 6.5 min (for 0.2 μM) to 150 min (for >100 μM). Reactions were stopped by boiling, and polyglutamate reaction products were resolved and quantified as described above. Kinetic parameters were subsequently determined by fitting to data the Michaelis-Menten equation using nonlinear, least-squares regression analysis (25).

***In vitro* inhibitor studies.** Inhibitor screening was performed using the standard assay (50 μM 4-NH_2-10-CH_3-Pte_{glu5}) and ~33 μunits of GGH/assay tube. Agents were tested at several concentrations of ≤1 mM when solubility permitted. More formal inhibition analyses were undertaken for the glutamine antagonists using at least seven concentrations of each compound and a 30-min preincubation of inhibitor with enzyme before commencing the assay by adding substrate. $K_{i\ app}$ values were obtained by fitting (25) to data the following equation: Inhibited Velocity = $V_{max}/\{1 + ([I]/K_{i\ app})\}$.

Analysis of polyglutamate chain formation. HT-1080 cells (2–5 × 10^7) were incubated in complete medium containing 10 μM [^{3}H]MTX at 37° for 24 hr or as described in text and figure legends. After the specified treatments, cells were harvested and suspended in 500 μl of boiling 50 μM Na phosphate, pH 5.5, and then placed in a boiling water bath for 5 min. Extracts were centrifuged at 20,000 × *g* for 10 min, and supernatants were analyzed by HPLC as previously described (26).

Results and Discussion

Activity and secretion of GGH from cultured cell lines. In all instances, the HPLC profile of GGH reaction products for assays (4-NH_2-10-CH_3-Pte_{glu5} as substrate) was consistent with an exopeptidase mode of action (Fig. 1A). This mode of activity is characterized by successive and sequential cleavage of end γ-glutamyl residues, with cleavage stopping at the one remaining glu residue (i.e., 4-NH_4-10-CH_3-Pte_{glu1} when standard substrate is used). We (28) and, more recently, others (29) have reported this activity for human GGH from cultured cell lines, although the possibility that other activity forms exist for other human cell types or tissues (e.g., tissues involved in absorptive processes) cannot be ruled out at this stage. In this study, a strict exopeptidase mode of action was also found for HT-1080 GGH at various levels of purity and for other poly-γ-glutamate substrates [i.e., Pte_{glu4} and pAB_{glu5} (*vida infra*)]. This clearly contrasts with data obtained from studies of many rodent tissues and rodent cultured cell lines. For these species, the enzyme has displayed either a mixed or an endopeptidase mode of cleavage for folate and antifolate poly-γ-glutamates (11, 19). Human GGH is therefore distinct from rodent GGH in this respect.

Similar to observations made by others in studies of rodent cell lines (19) and the (human) MCF-7 cell line (29), a significant level of GGH is secreted into the media during culture of HT-1080 cells. The media fraction of GGH activity increases as cells reach confluence and is markedly stimulated by the addition of NH_4Cl to the culture (Fig. 1B). This agent, at a millimolar concentration, inhibits the translocation of newly synthesized precursor protein destined for lysosomes and facilitates secretion into the extracellular media (30, 31). The activity of β-hexosamidase, a lysosomal marker enzyme (23), was also secreted in parallel with HT-1080 GGH, supporting the contention that secreted GGH came from the lysosomal compartment (data not shown). Conversely, the activities of cytosolic enzymes, glyceraldehyde-3-phosphate dehydrogenase, and dihydrofolate reductase in the media remained low (or below detection for the latter) throughout the duration of growth for both control and NH_4Cl-treated cells. Secreted HT-1080 GGH also displayed an exopeptidase mode of activity on assay.

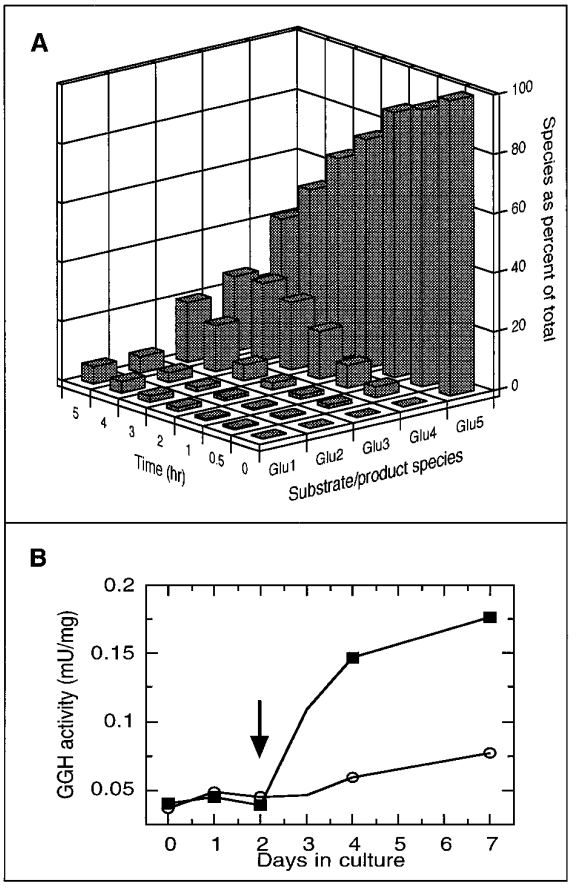

Fig. 1. Profile of GGH reaction products and secretion of enzyme from HT-1080 cells. A, Substrate 4-NH_4-10-CH_3-Pte_{glu5} (i.e., MTX_{glu4}) was incubated with HT-1080 GGH and then the assay mix was periodically sampled and products were resolved by reverse-phase liquid chromatography. Glu_5–Glu_1, derivatives of 4-NH_4-10-CH_3-Pte. This time course for substrate/product profile is consistent with an exopeptidase mode of action. B, Medium from cultured HT-1080 cells at early log growth was periodically assayed for GGH activity over 7 days either (■) with or (○) without the addition of 10 mM NH_4Cl at day 2 (*arrow*).

Purification of GGH from HT-1080 cells. GGH bound avidly to an agarose-immobilized dye matrix (Reactive Green 5) at pH 7.5 and could be eluted as a sharp band of activity by application of a salt gradient. Multiple protein components were detected on SDS-PAGE analysis of the eluted fractions with highest specific activity, nevertheless, this proved to be an ideal first step in the purification of GGH with good yield (51–82%, $n = 4$) and substantial purification (110–386-fold, $n = 4$). Further purification of HT-1080 GGH was achieved by passage on a organomercurial column (thiol elution) and Sephacryl-200 chromatography as summarized in Table 1. For both of these steps, activity behaved homogeneously (i.e., eluted as a single peak); however, omission of thiols from the S-200 equilibration buffer (in initial runs) resulted in an extremely broadly eluting activity peak. In addition, total

TABLE 1
Purification of GGH from the HT-1080 fibrosarcoma cell line

	Specific activity[a]	Fold purification (cumulative)	Yield for individual step
	units/mg		*%*
Crude cytosol	0.002		100
Reactive Green 5 chromatography	0.77	386	72
Dialysis			83
Organomercurial agarose	3.6	1800	68
Sephacryl S-200[b]	~6.0	~3003	21

[a] One unit is defined as the amount to convert 1 μmol of MTX glu_5 to lower polyglutamate forms (at 37°) in 1 min.
[b] Recoveries and GGH elution profile were dramatically affected by the presence or absence of thiols in applied sample and running buffer. The small quantities of protein available for total protein measurement made calculation of specific activities difficult.

activity recovered from the gel filtration step (see Table 1), and stability of the resulting preparation was low even when thiols and Zn^{2+} were present.

Analysis of the most highly purified preparation by SDS-polyacrylamide gel electrophoresis and silver staining revealed four readily discernible bands (and at least two faint bands), so the extent of purification necessary to obtain completely pure enzyme is unknown at this stage. Subsequent discussions in this article concerning the properties of human GGH refer to material of specific activity >3.0 units/mg (i.e., purified ≥1500-fold from crude extract) unless otherwise stated.

Properties of HT-1080 GGH. Concentrated partially pure GGH was stable at −75° for a 6-month period and demonstrated only ~6% activity loss after five freeze (−196°)/thaw (37°) cycles. Several general properties in relation to GGH activity were noted. (a) Activity was highly pH dependent with an acidic optimum in the range of 4- 6.5 (Fig. 2A). This is a similar finding to that reported for murine and bovine species of GGH (12, 19) and consistent with its assumed lysosomal localization. (b) Although activity was routinely assayed with buffer containing $ZnCl_2$ and DTT, omission of DTT often diminished activity by 1–15%. In no instance did omission of $ZnCl_2$ result in lower activity, even with the purist preparations and after exhaustive dialysis. When activation by DTT (2 mM) was apparent, an equal extent of activity enhancement could be obtained with β-mercaptoethanol or cysteine but not cystine or glutathione (data not presented). When the MTEN buffering component was replaced by citrate, succinic, and 2-(*N*-morpholino)ethanesulfonic acid buffers, each at 50 mM, pH 4.5, and also containing DTT and $ZnCl_2$, identical catalytic rates were observed. (c) GGH was inhibited by anions or polyanions as previously noted for other species of enzyme (2), presumably by binding a putative polyglutamate anion receptor site on the enzyme (18). Activity was completely abolished by 2 mM and 1 mM Na acetate at pH 4.5 and 5.5, respectively.

Physicochemical properties. The molecular mass of native HT-1080 GGH was estimated on four occasions by gel filtration chromatography on Sephacryl S-200 in the presence of 2 mM β-mercaptoethanol and 1 mM DTT. Values of apparent molecular mass obtained by interpolation of the calibration plot (Fig. 2B) ranged from 81.0 to 93.9 kDa; the average was 83.0 ± 9.6 kDa (±1 standard deviation). Omission of thiols from buffer resulted in a spreading of activity elution on initial trials, a phenomenon that did not seem to be related to the purity of material analyzed. This molecular mass estimate for HT-1080 GGH differs considerably from those reported for the partially pure jejunal enzyme [75 kDa, (10)], bovine liver GGH [108 kDa, (12)], and the secreted rat hepatoma enzyme [120 kDa, (19)]; each determined by gel filtration methodologies. For the latter enzyme species, more recent studies (8) with pure preparations (without deglycosylation) indicated a diffuse band of 55 kDa on SDS-polyacrylamide gel electrophoresis analysis, which is suggestive of quaternary structure for at least this rodent GGH species. Quaternary structure, extent of glycosylation, and how these factors contribute to differing apparent molecular mass estimates for various eukaryotic sources of GGH have yet to be determined.

Isoelectric focusing of HT-1080 GGH (3.6 units/mg) in the presence of urea and Nonidet P-40 gave rise to one major activity band at pI 6.2–6.4 (representing 64% of the total detected activity) and two other regions of activity; one corresponding to pI 5.6–6.6 and the other at 7.3–7.8 (Fig. 2C). The latter, representing 18% of total detectable activity, may simply be the result of aggregated enzyme collected at the agarose loading/acrylamide interface. Nevertheless, the presence of at least two regions of activity represents the only observation of activity heterogeneity in the current study. Apart from the possibility that these isoforms represent proteins of different primary structure, several other possibilities exist, including the dissociation of quaternary structure (as discussed above) and/or variations of post-translational modification of a single protein species. The latter possibility seems most likely in view of the strong possibility that the enzyme is glycosylated due to its lysosomal location. No other report of the behavior of GGH on isoelectric focusing has been described to our knowledge.

Enzyme kinetic parameters and substrate specificity. The catalytic activity of HT-1080 GGH was measured with varied concentrations of Pte_{glu2}, 4-NH_2-10-CH_3-Pte_{glu2}, pAB_{glu2}, and the corresponding glu_5 derivatives. The amount of enzyme and the time points selected in assays were chosen to avoid substantial substrate depletion and to permit the generation of predominantly glu_4 product derivatives only in the case of glu_5 substrates. All substrates conformed to Michaelis kinetics, yielding rectangular hyperbolic curves when reaction velocities were plotted against substrate concentration. Iterative fitting of the Michaelis-Menten equation to data enabled estimation of K_m and V_{max} parameters, as listed in Table 2. Most notably, the enzyme showed preference toward the longer-chain derivatives with 15–42-fold lower K_m values for the glu_5 versus glu_2 forms for each of the substrate types analyzed. A similar observation of distinct preference for the longer-chain γ-glutamate substrates has been noted in other studies (some are reviewed in Ref. 2), including the early work with partially pure human liver enzyme [although only in a qualitative fashion (18)] and more recent work by Sirotnak *et al.* (11) with murine GGH. In the latter study, analyses of GGH activity in crude preparations of L1210 and mouse small intestine revealed respective 3- and 7.7-fold decreases in K_m values for the glu_4 derivatives of 4-NH_2-10-CH_3-Pte versus the glu_2 derivative (11). It is also worthy of note that in this same study (11), enriched GGH

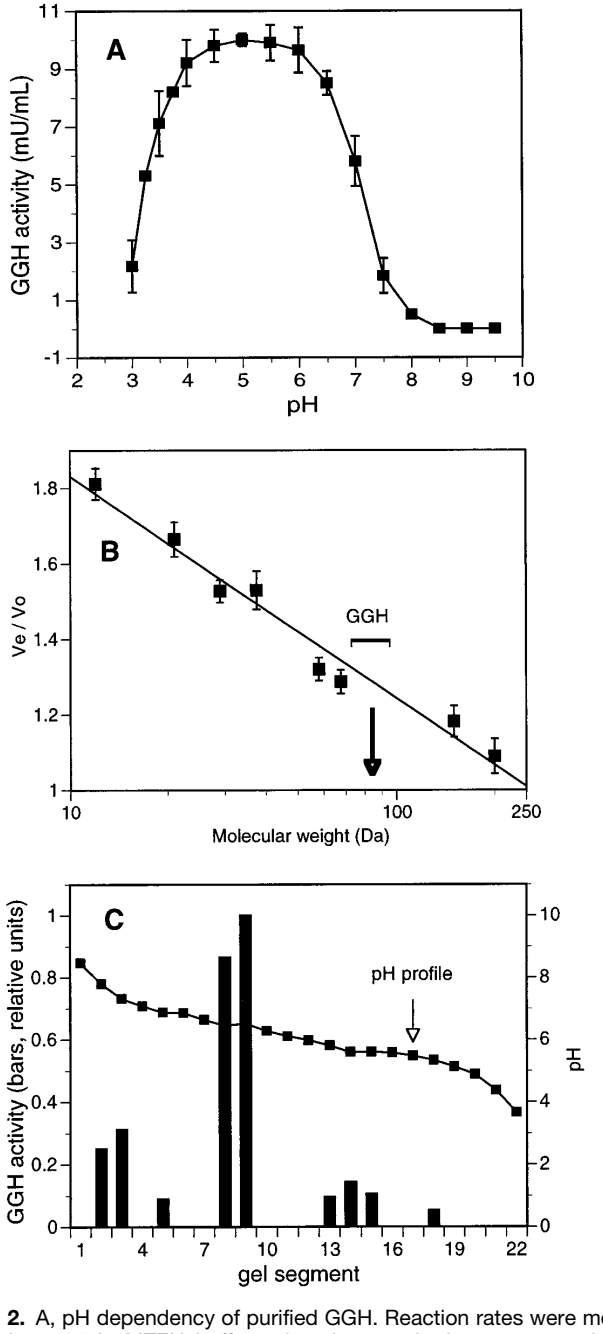

Fig. 2. A, pH dependency of purified GGH. Reaction rates were measured at 37° in MTEN buffer using the standard assay procedure. Individual data *points* represent the average activity (±1 standard error) for three independent measurements. Appropriate assay controls were included to ensure that the lower activities measured at pH extremes reflect genuine reduced catalytic ability rather than enzyme inactivation. B, Estimation of the molecular mass of native GGH by gel filtration. Partially pure GGH was applied to a column of S-200 gel previously equilibrated with a buffer consisting of 20 mM Tris·HCl, pH 7.0, 10 mM NaCl, 2 mM β-mercaptoethanol, and 1 mM DTT. Enzyme activity was eluted with the same buffer. Standard proteins used to calibrate the column are depicted (■), as is the activity elution position of GGH from several runs. The interpolated average molecular mass for GGH is 83.0 ± 9.6 kDa. C, Isoelectric focusing of GGH. Partially pure enzyme was focused in a tube gel (polyacrylamide; 4% T, 0.5% C) containing carrier ampholytes pH 3.5–10 (2%) and pH 5–7 (3.1%). Focusing conditions and activity determinations were carried out as described in Experimental Procedures. The pH gradient of the gel was calibrated by measuring pH of macerated gel segments (dissolved in minimal water) from a second blank gel ran in tandem. The three major activity regions correspond to pI values of 6.2–6.4, 5.6–5.8, and 7.3–7.8.

TABLE 2
Kinetic parameters for purified sarcoma GGH

Substrate glu chain length (n)	$Pte_{glu\,n}$	4-Amino-10-methyl-$Pte_{glu\,n}$	$pBA_{glu\,n}$
$Glu_2 \rightarrow glu_1$			
K_m (μM)	16.3 ± 1.4	52.6 ± 5.3	59.3 ± 3.4
$^aV_m^{rel}$	0.86	0.97	1.11
$glu_5 \rightarrow glu_4$			
K_m (μM)	0.9 ± 0.1	3.4 ± 0.6	1.4 ± 0.2
$^aV_m^{rel}$	0.99	1.00	0.94
Activity ratio[b]	20.8	15.9	35.8

[a] Maximum velocity relative to 4-amino-10-methyl-Pte $glu_5 \rightarrow glu_4$ derivative.
[b] Catalytic efficiency (V_m/K_m) for longer-chain substrates ($n_5 \rightarrow n_4$) versus shorter-chain substrates ($n_2 \rightarrow n_1$). Note: Pte_{glu1} is folic acid.

from mouse sarcoma 180 cells and mouse intestine extract (through ammonium sulfate fractionation) displayed K_m values of 19.7 and 86.4 μM, respectively, for 4-NH_2-10-CH_3-Pte_{glu2}. These values encompass the value obtained in the current study with human enzyme using identical substrate (52.6 μM; Table 2).

The HT-1080 enzyme had little regard to the presence or absence of the pteroyl group for substrate binding; this is more pronounced for the longer poly-γ-glutamate substrate, where the K_m value for pAB_{glu5} was only 1.6-fold higher than that for Pte_{glu5} (Table 2). The partially pure human liver GGH was also found to have qualitatively good activity with either Pte_{glu4} or γ-glu_4 as substrate (18, 32). Combined, these data strongly argue against the existence of a Pte-specific binding site on the surface of human GGH, although it is interesting to note that the 4-NH_3-10-CH_2-Pte modification of Pte_{glu2} and Pte_{glu5} resulted in a 3.2- and 3.8-fold reduction in affinity, respectively (Table 2). Also of significance is the fact that the maximal possible catalytic activity for each substrate examined (i.e., V_{max}; Table 2) is approximately equal , so differences in overall catalytic efficiency for substrates are primarily due to binding considerations.

The properties of substrate specificity for HT-1080 reported here seem to reflect a general consensus of properties for all eukaryotic species of GGH, regardless of the mode of reaction (endo- or exo-) catalyzed. One exception to this may be the chicken intestine enzyme, which was reported to be active toward Pte_{glu5} but not Pte_{glu3}, γ-glu_5, or *N*-trinitrobenzoyl-γ-glu_5 (2, 33). Interestingly, the dipeptide analog of the quinazoline antifolate ICI 198583 (2-desamino-2-methyl-N^{10}-propargyl-5,8-dideazafolic acid), which has a D-enantiomeric glutamic acid residue appended at the γ-position of the folyl L-glutamate [i.e., ICI 198583-γ-D-glu (34)], did not serve as a substrate (data not presented), indicating binding stereoselectivity for the human GGH active site.

Inhibition of HT-1080 GGH. 2-Mercaptomethylglutaric acid was reported to be inhibitory toward partially pure chicken pancreas GGH but not lysosomal hog kidney GGH (17, 35). Experiments performed in the current study with HT-1080 GGH failed to show any *in vitro* inhibition of activity by this agent at ≤250 μM, at least at pH 4.5. To identify inhibitors, we used initially the quantitative HPLC assay and later the high through-put capillary electrophoresis assay to screen a series of potential inhibitor compounds selected on the basis of structural considerations. Many of these compounds were folate or antifolate derivatives with unusual glutamate modifications or substitutions, although

none selected displayed significant inhibition,[1] including ICI 198583-γ-D-glu. However, the "glutamine antagonists azaserine, acivicin, and DON, all of which are well-characterized inhibitors of γ-glutamyltranspeptidase [EC 3.3.2.2 (36)], were found to be inhibitory toward the human GGH. Without regard to the mode of inhibition, or the presence of substrate, and using inhibition reaction rates after a 30-min preincubation with enzyme, respective $K_{i\ app}$ values of 5.7, 0.85, and 0.05 mM were derived for these agents (Fig. 3A).

As mentioned, each of these agents is a potent inhibitor of γ-glutamyltranspeptidase, an enzyme that catalyzes the transfer of the γ-glutamyl of glutathione and other γ-glutamyl compounds to a number of acceptors (36). The rationale for testing these antagonists against GGH was therefore based on the possibility of similar active site architecture for the two enzymes because both catalyze the cleavage of a γ-glutamyl moiety. It has also been established that each of these compounds binds irreversibly to the γ-glutamyltranspeptidase active site, and although formal experiments have not been undertaken to determine whether covalent modification also occurs for GGH, it is interesting to note that time dependency for DON inhibition is observed (Fig. 3B), a phenomenon that is frequently observed for irreversible enzyme inhibitors.

Cell culture studies of GGH inhibition by DON and its effect on MTX action. The cytotoxicity of DON toward the HT-1080 line by DON alone was determined before assessing the potential of this agent to enhance MTX retention and action. Cell survival after a 24-hr exposure to DON at concentrations of ≤500 μM was measured using the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino)carbonyl]-2*H*-tetrazolium hydroxide cytotoxicity assay. For the HT-1080 cell line, < ~5% cell death was observed at 10 μM DON, so this was the maximal concentration adopted in subsequent studies.

We then examined the effect of DON on *in situ* GGH activity. Cultured cells (HT-1080 and HS-16, at mid-log growth phase) were incubated with DON at 0, 5, or 10 μM for 24 hr; then, cells were harvested, and GGH activity was measured for clarified extracts. Because harvested cells were extensively washed before lysis, any inhibition of GGH was interpreted as the result of intracellular action of DON. Compared with the GGH activities measured for conditions where DON was absent, GGH activities for the HT-1080 line were 80% and 89% suppressed, and the HS-16 line was 89% and 94% suppressed for the 5 and 10 μM DON exposures, respectively.

A more direct analysis of the effect of DON on cellular polyglutamylation proficiency was obtained by simultaneous exposure of cells to radiolabeled MTX and subsequent analysis of the polyglutamylation profile. As indicated in Table 3, total MTX accumulated (all polyglutamate species) was comparable within each cell line examined regardless of whether DON was present or absent. However, the percentage of MTX present as the longer glu chain forms (i.e., ≥glu_3) was higher for each of the cell lines when DON was also present (1.8- and 3.3-fold higher for HT-1080 and HS-16, respectively). Such altered polyglutamylation profiles are consistent with GGH inhibition.

[1] A list of screened compounds and activities is available from the authors on request.

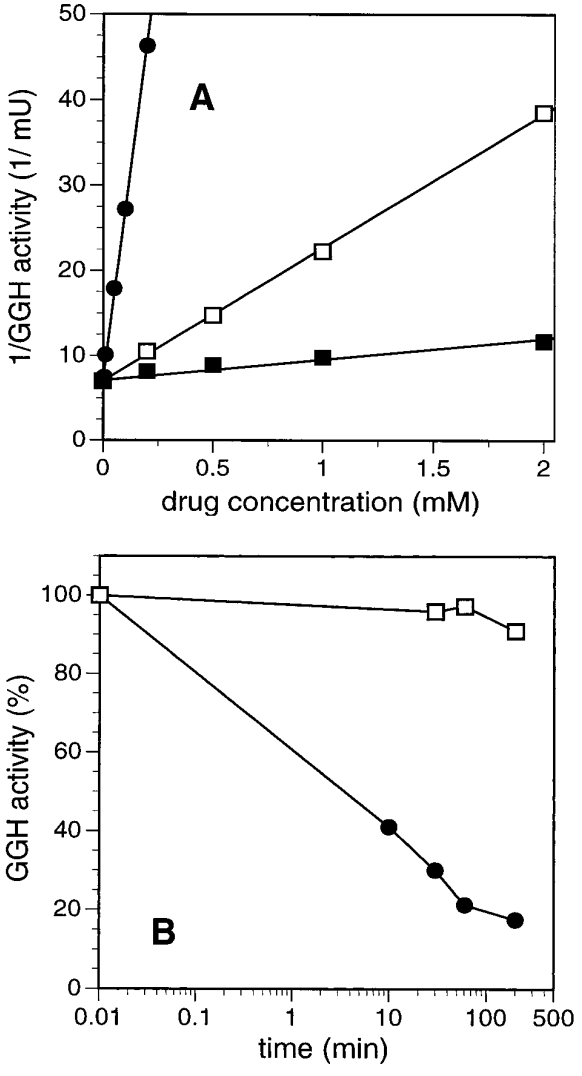

Fig. 3. *In vitro* inhibition of purified GGH. A, Dixon plots of data obtained for (■) azaserine, (□) acivicin, and (●) DON inhibition of HT-1080 GGH. Different concentrations of agent were preincubated with enzyme for 30 min before assessment of activity using the standard GGH assay protocol (substrate 4-NH_2-10-CH_3-Pte_{glu5} at 50 μM). B, Time dependency for inhibition by DON. GGH (30 μunits) was incubated with 50 μM DON for varying times at 37° and then assayed as above (●). Inhibition data are presented as the percentage value of control activity measurements for which preincubation was carried out without added agent (□).

An experiment was undertaken to examine possible cell-kill synergism of MTX and DON (Table 4). For the two sarcoma lines examined (HT-1080 and HS-16), enhanced cell kill was obtained for MTX when DON was also present at a concentration previously demonstrated to be insignificantly toxic (i.e., 10 μM). This was most dramatic for the HS-16 line with an IC_{50} value 9-fold lower when DON was present as opposed to MTX alone.

In addition to the GGH inhibition by DON described in the current work, this compound has many defined sites of pharmacological action, including γ-glutamyltranspeptidase [as mentioned previously, (36)] and several glutamine dependent amido transferases involved in nucleotide biosynthesis (37). Thus, other cellular perturbations besides those resulting from GGH inhibition are possibly occurring, and this factor

TABLE 3

Effect of DON on intracellular MTX polyglutamate profile in sarcoma cells

HT-1080 and HS-16 cells were incubated with [^{3}H]MTX (10 μM) with or without simultaneous exposure to DON (10 μM). After 24 hr, cultures were trypsinized and washed, and cells were counted. Extracts then were prepared, and MTX metabolites were analyzed by HPLC. Polyglutamate quantities are presented per cell number.

Cell line	Polyglutamate chain length (*n*)					Total	Longer chain length[a]
	1	2	3	4	5		
	pmol/10^7 cells						*%*
HT-1080							
+MTX	68.4	11.2	21.6	11.6	6.5	120.0	33.3
+MTX and DON	43.6	5.2	30.2	22.5	17.0	118.5	59.9
HS-16							
+MTX	62.5	1.2	2.4	1.5	1.0	68.6	7.1
+MTX and DON	58.7	5.4	6.5	7.4	5.3	81.5	23.6

[a] The sum of glu_3, glu_4, and glu_5 [^{3}H]MTX metabolites as a percentage of all [^{3}H] metabolites detected.

TABLE 4

Effect of DON on growth inhibition by MTX in soft tissue sarcoma cell lines

Cultured cells were exposed to varied concentrations of MTX (1 nM to 10 μM) with or without simultaneous exposure to DON (10 μM). After a 24-hr exposure, cells were washed, and drug-free media were replaced. At an additional 96 hr later, single suspensions were prepared for each condition, and cells were counted. IC_{50} values were interpolated from cell survival plots.

Cell line	IC_{50}	
	MTX	MTX + DON
	nM	
HT-1080	181	67
HS-16	2413	262

must warrant consideration when interpreting data presented in Tables 3 and 4.

Concluding comments. The properties of purified human GGH reported in the current study have similarities to the few characteristics noted for human liver GGH but differ in several respects to those reported for rodent, bovine liver, and human jejunal and brush border enzymes. GGH for cultured human cell lines (excluding those derived from tissues with absorptive functions) may be predominantly one species, an exopeptidase that is highly sequestered into the lysosome. The only indication of possible GGH variants identified in the current work could also be explained on the basis of variable post-translational modification of the enzyme (i.e., glycosylation).

Consideration for the potential of GGH inhibition as a therapeutic strategy in conjunction with antifolate exposure has been previously described (17). Some additional support for this strategy comes from reports of several eukaryotic cell lines with increased GGH activity that are resistant to MTX and/or other antifolate drugs (28, 38, 39) and the current view that the majority of patients with acute myelogenous leukemia are intrinsically resistant to MTX due to impaired polyglutamylation (and therefore retention) of the drug (27, 40). The results presented here provide some preliminary detail on the physicochemical and catalytic properties of this candidate target. DON and acivicin may serve as useful lead compounds in the development of specific GGH inhibitors. Furthermore, such agents should prove useful in exploring the relationship between GGH activity levels and antifolate resistance observed in experimental systems and in the clinic.

While this article was being reviewed for publication, Yao *et al*. (41) published the sequence and *in vitro* expression of human GGH. The cDNA encodes for a polypeptide of ~36 kDa with four consensus asparagine glycosylation sites. Their preliminary characterization studies of expressed protein (including substrate preference) is consistent with those reported here for native enzyme.

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