ELSEVIER

Journal of Chromatography B, 689 (1997) 387–392

JOURNAL OF CHROMATOGRAPHY B

Capillary electrophoresis of methotrexate polyglutamates and its application in evaluation of γ-glutamyl hydrolase activity

Mark C. Waltham[1], Silan Lin, Wei-Wei Li, Erdem Göker, Helena Gritsman, William P. Tong, Joseph R. Bertino*

Program of Molecular Pharmacology and Therapeutics, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021, USA

Received 15 February 1996; revised 5 July 1996; accepted 26 July 1996

Abstract

A rapid and sensitive procedure for the separation of methotrexate (MTX) polyglutamates[2] using capillary electrophoresis (CE) is described as it applies to the in vitro assay of the enzyme γ-glutamyl hydrolase (GGH, EC 3.4.22.12). Distinct separation of MTX and polyglutamylated forms (up to glu_4) is achieved within 10 min using a 75 μm I.D. capillary (50 cm, +25 kV), and enables quantitation of both reactant and enzyme products. As activity can be reliably determined using less than 5×10^5 eukaryotic cells, this new technique can be used to measure GGH in patient tumor samples and investigate the relationship between GGH levels and clinical MTX resistance.

Keywords: Methotrexate polyglutamates; γ-Glutamyl hydrolase; 4-Amino-10-methylpteroylglutamate

1. Introduction

MTX is an antifolate drug used in the therapeutic treatment of a variety of neoplastic and proliferative diseases, although its clinical effectiveness is frequently hampered by intrinsic and acquired cellular resistance [1]. After gaining access to the cell, MTX is modified to polyglutamylated derivatives which are preferentially retained for longer duration than MTX itself [2]. Folylpolyglutamate synthetase (EC 6.3.2.17) is the enzyme which catalyzes the successive addition of γ-glutamyl groups to the γ-carboxyl of MTX (creating a more hydrophilic form of the drug). Conversely, the predominately lysosomal enzyme, γ-glutamyl hydrolase (GGH; but also referred to as folylpolyglutamate hydrolase or conjugase), cleaves added γ-glutamyl groups via either an exo- or endopeptidase activity, depending on species and tissue (Fig. 1). The activities of both FPGS and GGH, in addition to the rate of intralysosomal import of MTX polglutamates, can therefore play a major role in the overall retention and potency of MTX for a given cell type. Indeed, several cell lines with increased GGH levels have been described which are MTX-resistant and resistant to other "new genera-

*Corresponding author.

[1] Present address: National Cancer Institute, NIH, Bldg. 37, Rm 5C-25, 9000 Rockville Pike, Bethesda, MD 20892-4255, USA.

[2] Note on nomenclature: MTX (methotrexate), 4-amino-10-methylpteroylglutamate; MTX + glu_1, + glu_2, + glu_3, + glu_4 refer to 4-amino-10-methylpteroyldiglutamate, triglutamate, tetraglutamate, heptaglutamate respectively; polyglutamates and + glu_n abbreviations imply γ-linkage of L-glutamyl residues.

PII S0378-4347(96)00350-7

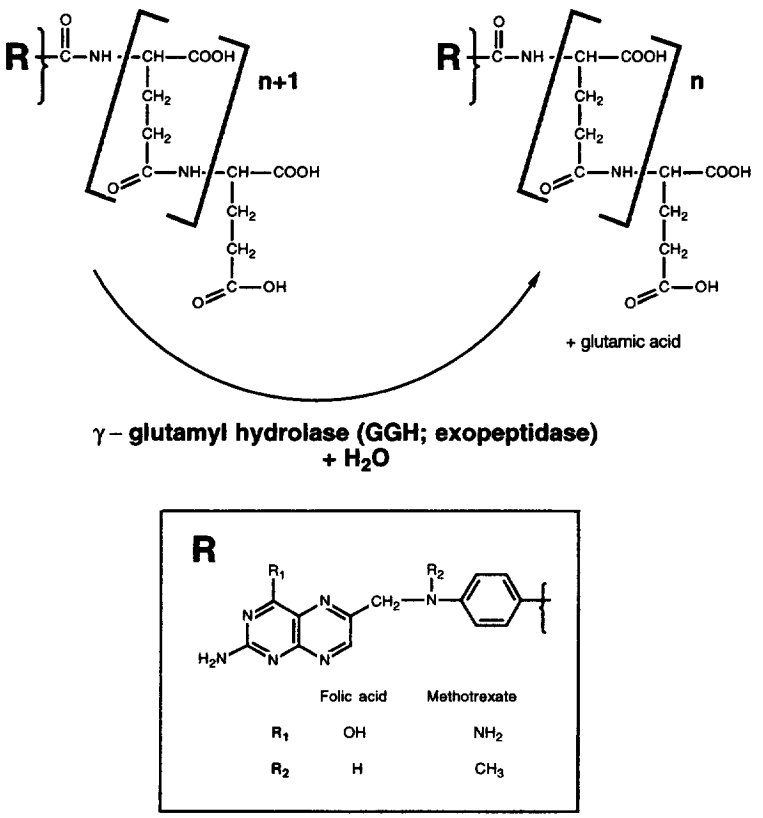

Fig. 1. The reaction catalyzed by GGH. This enzyme hydrolyzes the γ-glutamyl linkages of folate and MTX polyglutamates (structures given in insert), as well as other folate/antifolate polyglutamate substrates. Exopeptidase species cleave successive residues (decreasing n incrementally), but only to $n=0$ (i.e. to folic acid or MTX).

tion" antifolate drugs, which also require polyglutamylation for effective retention [3–5]. Despite this, little information is available concerning GGH in terms of tumor expression levels, nor of its potential role in contributing to clinical forms of MTX resistance.

A colorimetric assay [8] and variations on a HPLC method [6,7] have previously been described for measurement of GGH activity. The latter methods permit quantitation of both reactants and cleavage products, but the colorimetric procedure does not, nor has it proven to be very sensitive in our hands. In order to investigate clinical tumor samples which are frequently only available in small quantity, we assessed an alternative assay for GGH using capillary electrophoresis (CE); an analytical technique which although considered less sensitive than HPLC (i.e. poor concentration limit of detection for CE), requires only a very small sample size [9]. Here we present a CE method for separation and quantitation of MTX polyglutamates for the evaluation of GGH activity. Application of the assay to determine GGH levels in (patient) leukemic blast samples and cultured cell lines is described.

2. Experimental

2.1. Chemicals and starting materials

Sodium tetraborate (borax), dithiothreitol (DTT), pepstatin A, aprotinin and phenylmethylsulfonyl fluoride (PMSF) were from Sigma (St. Louis, MO, USA). Leupeptin came from U.S.B (Cleveland, OH, USA). Sodium dodecyl sulphate (SDS, "certified" grade) was from Fisher Scientific (Pittsburgh, PA,

USA). Polyglutamate compounds were purchased from Dr. B. Schirks Laboratories (Jona, Switzerland) except for MTX which was from Lederle Laboratories (Pearle River, NY, USA). All solvents and other reagents were HPLC grade. Electrophoresis buffers were filtered through Millipore (Bedford, MA, USA) membranes (0.45 μm) prior to use. Various cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA).

Cell lines were maintained under standard conditions (RPMI-1640 media containing 10% fetal calf serum, 2 m*M* glutamine, antibiotics at 37°C and in 5% CO_2) and harvested at mid-log growth by trypsinization and subsequent extensive washing with phosphate buffered saline.

Patient blast samples were obtained from bone marrow aspirates or peripheral blood after obtaining informed consent, and processed as described previously [10]. All patients at the time of sampling had greater than 80% blasts and, after Ficoll–Hypaque separation, cells had >90% viability by trypan blue exclusion. As for cultured cell lines, washed cells were either lysed immediately for GGH assay or snap-frozen as a wet cell pellet in liquid N_2, then stored (−75°C) for later use.

2.2. Preparation of lysates and assay conditions

Typically, 5×10^6 cells (cultured cells or blasts) were resuspended in 0.5 ml of 50 m*M* sodium phosphate (pH 7.0) containing DTT (2 m*M*), PMSF (0.1 m*M*), aprotinin (500 KIU/ml), pepstatin (1 mg/ml) and leupeptin (1 mg/ml). Complete lysis was achieved by six 20-s sonication bursts using a micro-sonication probe or an inverted bath sonicator for volumes less than 500 μl. Suspensions were then clarified by centrifugation at 100 000 *g* for 15 min (at 4°C), and the resulting supernatant recovered for immediate analysis.

Ten to 20 μl of extract was incubated with substrate (MTX+glu_4) in MTEN buffer [11], pH 4.5, containing $ZnCl_2$ (1 m*M*) and DTT (2 m*M*) at 37°C with gentle shaking. Portions of the assay mix were sampled at various time points, at which time they were placed in a boiling water-bath (5 min), then spun at 14 000 *g*. A portion of the supernatant was then directly diluted into 100 to 150 μl of water contained in an appropriate CE insert vial. Samples were analyzed immediately by CE or stored at −20°C (protected by light) and run at a later date. In the case of the latter, the stability of assay products was confirmed in several instances by periodic analysis of stored sample. Total protein concentration for lysates were obtained by Bradford analysis [12] using bovine serum albumin as standard.

2.3. Instrumentation and separation

An Thermo Separation SpectraPhoresis 1000 CE unit (Fremont, CA, USA) was used in all analyses. Separation of polyglutamates was performed in untreated silica capillary tubes (Polymicro Technology, Phoenix, AZ, USA) of dimensions 75 μm I.D. (375 μm O.D.) by 50 cm to electrode (44 cm to detector). Running voltage for separation was set at +25 kV, temperature at 25°C and typical running current was ~85 μA. The vacuum level for both washing and injection procedures was 10.3 kPa. The routine running buffer selected was 20 m*M* sodium tetraborate with 20 m*M* SDS at pH 9.5. Vacuum injection mode set at 2 to 9 s, was used and detection was at 300 nm. Wash sequence was between each sample injection: 0.1 *M* NaOH for 1 min (50°C), water for 1.5 min (50°C) followed by running buffer (borax/SDS) for 2.5 min (25°C). Standard software accompanying this instrument permitted quantitation of eluting peaks, however, no correction was made for the fact that peaks arriving at the detector are travelling at different velocities [13,14]. The slight overestimate of quantities for later-migrating peaks should be avoided in future studies by using time-normalized peak areas (i.e. A/t_m, [14]) in calculation of activity measurements (below).

2.4. Activity calculation

The amount of GGH activity for a given sample is calculated from the percentage of MTX+glu_4 degraded per unit time according to the following equation:

$$[(\alpha - \text{area under peak for MTX} + \text{glu}_4)/\alpha]/\text{time}$$

where α = sum of area under peaks for MTX, MTX+glu_1..... MTX+glu_4.

Taking into account the volume of the original

assay mix, and the concentration of substrate, this value is converted to Units (U) per ml of sample analyzed, where one U is defined as the amount required to convert 1 μmol of substrate per min. Specific activity values (U/mg protein) for patient and cell culture samples were calculated from assay time points where less than 30% of substrate was consumed.

3. Results and discussion

GGH during substrate incubation is buffered at pH 4.5 as the enzyme has an acidic pH optimum (complementing its lysosomal localization), and because folylpolyglutamate synthetase is inactive at this pH [2]. A typical CE electropherogram of resolved products obtained from the incubation of MTX+glu_4 (50 μ*M*, standard substrate and concentration) with crude extract of a human T-cell leukemia cell line (CEM cells) is displayed in Fig. 2. The identity and order of elution of MTX polyglutamates were confirmed using mixtures of standards prepared from the commercial source. This profile is consistent with an exclusive exopeptidase mode of action for the human GGH (i.e. successive removal of terminal γ-glutamyl residues), although, curiously, the amount of MTX detected in reaction products

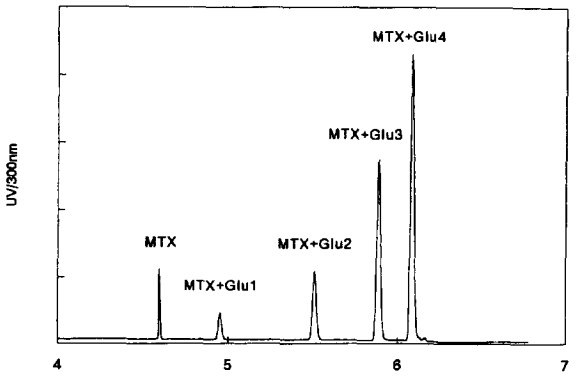

Fig. 2. CE electropherogram of GGH reaction products. Substrate (MTX+glu_4) was incubated with CEM extract, then assay products processed and resolved in a capillary tube (+25 kV, micellar electrokinetic mode), as described in Section 2. Both remaining substrate and reaction products are quantitated. This substrate/product profile is consistent with an exopeptidase mode of action. The abscissa represents time (in minutes).

was frequently similar to, or slightly greater than, that for MTX+glu_1. As blank assay samples (where substrate was incubated without added crude extract) displayed the MTX+glu_4 peak only, this phenomena can not be attributed to MTX contamination of substrate, nor the generation of MTX by non-enzymatic means at 37°C. Additionally, this phenomena is also observed on HPLC analysis of human GGH assay products (not presented). Moreover, the general features of the reaction product profile depicted in Fig. 2 (indicating exopeptidase activity) were observed for all samples analyzed during the course of this work, including patient material. As we have previously noted from studies employing the HPLC assay [4], these profiles contrast with those observed for murine species of enzyme which conform to either an endopeptidase or "mixed" mode of catalytic activity [3,6].

The mode of CE separation used in this study is known as micellar electrokinetic capillary chromatography. A detailed description of the separation principle is given in Ref. [9]. Under the defined conditions, polyglutamates are well resolved and elution is complete within 10 min. Washing and re-equilibration steps conducted between each injection of sample permitted injections at 15-min intervals. Partial deproteinizing the sample by boiling (after enzyme incubation), then clarifying by centrifugation, presumably aided capillary longevity and reproducibility of separation. In fact, no alteration in product resolution was observed when >500 samples were analyzed using a single capillary tube. The speed and cost per analysis is, therefore, considerably better than the reversed-phase HPLC methods previously described [6,7]. Using an automated injector mode, we have now established a high-throughput assay to screen various compounds for in vitro inhibition of GGH. A potent GGH inhibitor would be useful to study the role of GGH in MTX action and to potentiate MTX cytotoxicity.

Using the CE separation methodology, routine assay parameters for GGH were tested using enzyme contained in crude extracts of CEM cells. In instances where adequate sample material was available ($n=8$), analysis of assay products by the conventional HPLC procedure [6] yielded GGH activity values (in mU) to within ±6% of those determined by the CE method described in the

current study. In Fig. 3A, the effect of enzyme concentration on amount of substrate degradation is presented. For the given quantities of added extract, the percentages of substrate consumed increase proportionally with time over a 4-h period. Furthermore, the rate of these reactions (the slopes of the lines) are proportional to the given volume of extract added to each assay tube. In the absence of enzyme, no apparent degradation of substrate was observed for the 4-h period under the given assay conditions of pH 4.5 and 37°C.

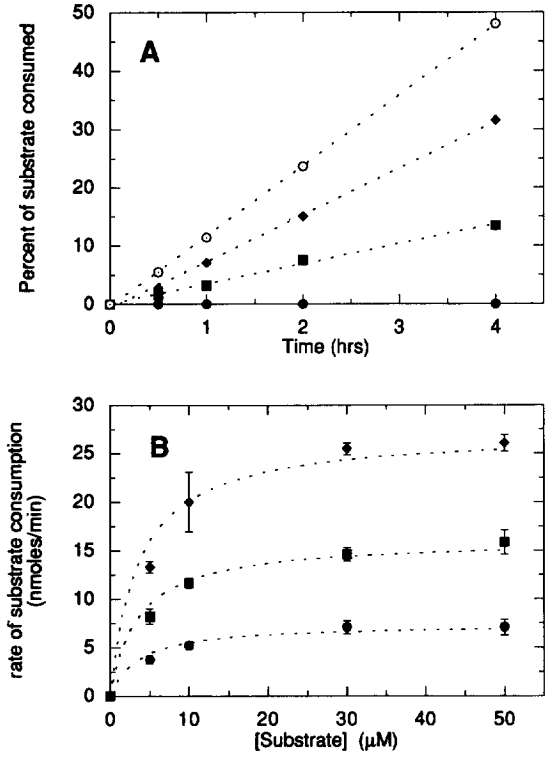

Fig. 3. Linearity of GGH assay (A) and dependence of substrate concentration on GGH activity (B). Panel A: different volumes of crude CEM extract [(●) 0 μl; (■) 5 μl; (♦) 10 μl; (○) 20 μl] were incubated with MTX + glu_4, and aliquots of the reaction mix sampled at various time points, then processed and analyzed by CE. Regression coefficients for each depicted line are >0.97. Slopes (i.e. reaction rates) are 0.0, 5.5, 12.2 and 19.4 (relative units), respectively, for the aforementioned volumes of extract. Panel B: triplicate measurements of GGH activity was measured (1×S.E. depicted) for three amounts of CEM extract at various concentrations of MTX + glu_4. Maximal activity is apparent at 50 μ*M* substrate and so this concentration has been adopted for routine assays.

Fig. 3B presents the effect of substrate concentration on the assay of GGH. Three different quantities of test extract were incubated for 1 h at varying concentrations of substrate. Maximal velocity for the assay was apparent at MTX + glu_4 concentrations greater than 30 μ*M*. In view of this, 50 μ*M* of MTX + glu_4 was adopted as the standard concentration used in assays.

As an important objective for developing this assay was to measure GGH levels in tumor samples derived clinically, we assessed the sensitivity of the CE based assay by performing a cell limitation study (Table 1). For this experiment, mid-log growth CEM cells were harvested, washed, then serially diluted at known cell numbers into 200 μl of assay buffer. After sonication and high-speed centrifugation, the supernatant was added to substrate and mixtures incubated at 37°C for 2 h. At the end of this period, samples were processed as described before. For assays containing less than 5000 cells, an insignificant quantity of substrate breakdown was apparent. For quantities greater than 10^5 cells, the percentage breakdown could be readily quantitated. For subsequent routine analyses, a volume of extract equivalent to 5×10^5 cells was used and portions of the 200-μl assay mix were sampled at various time points. Only a very small volume for CE injection is required (ca. 10 nl) for analysis.

Using the procedure described above, the GGH activity in several leukemia blast extracts and cultured cell lines was determined. As depicted (Fig. 4),

Table 1
Cell limitation study

Cell number	Conversion of substrate[a] (MTX + glu_4) (%)	Activity (mU per 10^6 cells)
0	NS	0
5000	NS	0
10 000	NS	0
50 000	~5.0	~82
100 000	9.4	78
500 000	43.9	73

Washed CEM cells were suspended in 200 μl of GGH assay buffer (MTEN buffer [11] at pH 4.5, containing 1 m*M* $ZnCl_2$ and 2 m*M* DTT) then lysed and assayed (for 2 h) as described in the text. Samples were subsequently boiled, centrifuged, then MTX and MTX polyglutamates resolved and quantitated by CE.

[a] NS: No significant conversion of substrate detected.

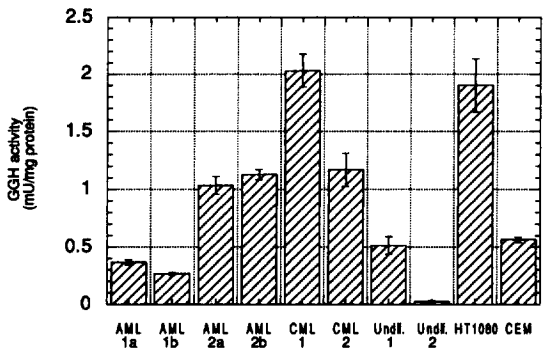

Fig. 4. GGH specific activities of patient and cell culture samples. Blast samples were obtained from acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML) and undifferentiated (undif.) leukemic patients. Further details are given in the text. Samples from CML patients were obtained during blast crisis. HT-1080 is a human fibrosarcoma cell line. CEM is a human T-cell leukemia cell line. Depicted errors represent 1×S.E. and incorporate errors associated with both GGH assay (triplicate) and protein estimate (triplicate).

a considerable variation in specific activities for this enzyme is apparent. Represented activity measurements for patients are from unrelated individuals except for (1) AML1a and 1b, which are activity measurements from blast samples derived from the same patient (peripheral blood) 24 h apart (AML1a "day 1", 0.37±0.02 mU/mg; AML1b "day 2", 0.27±0.01 mU/mg) and (2) AML2a and 2b which are also from an individual patient: AML2a (1.03±0.08 mU/mg) for blasts obtained from peripheral blood and AML2b (1.12±0.04 mU/mg) for blasts derived from a bone marrow aspirate (within 6 h of the former). The closeness of these assay values and associated small error estimates (derived from triplicate protein and GGH assay measurements) give support for the potential of this assay in clinical measurement.

We are currently investigating blast samples obtained from patients pre- and post-chemotherapy for regimens which have included antifolates. Together with determined activity measurements for folylpolyglutamate synthetase in these extracts, these analyses may shed light on the observations that variable antifolate polyglutamylation potential exists for different tumor varieties. In addition, and in particular for the leukemias [15–17], we wish to investigate the relationship between GGH activity levels and clinically observed MTX resistance.

Acknowledgments

This work was supported by grants from the United States Public Health Service (CA08010) and the American Cancer Society (ACS DHP-18-J).

References

[1] J.R. Bertino, J. Clin. Oncol., 11 (1993) 5.
[2] J.J. McGuire and J.K. Coward, in R.L. Blakely and S.J. Benkovic (Editors), Folates and Pterins, Vol. 1, Wiley Interscience, New York, 1984, p. 135.
[3] M.S. Rhee, Y. Wang, M.G. Nair and J. Galivan, Cancer Res., 53 (1993) 2227.
[4] W.W. Li, M. Waltham, W. Tong and J.R. Bertino, Adv. Exp. Med. Biol. Chem., 338 (1993) 635.
[5] G. Pizzorno, B.A. Moroson, A.R. Cashmore, O. Russello, J.R. Mayer, J. Gallivan, M.A. Bunni, D.G. Priest and G.P. Beardsley, Cancer Res., 55 (1995) 566.
[6] L.L. Samuels, L.J. Goutas, D.G. Priest, J.R. Piper and F.M. Sirotnak, Cancer Res., 46 (1986) 2230.
[7] Y. Wang, R.F. Rotundo, Z. Nimec, T.J. Ryan and J. Galivan, Adv. Exp. Med. Biol., 338 (1993) 655.
[8] P.V. Wagh and T.I. Kalman, Anal. Biochem., 207 (1992) 1.
[9] S.F.Y. Li, Capillary Electrophoresis: Principles, Practice and Applications, Elsevier, Amsterdam, 1992.
[10] E. Göker, M. Waltham, A. Kheradpour, T. Trippett, M. Mazumdar, Y. Elisseyeff, B. Schnieders, P. Steinherz, C. Tan, E. Berman and J.R. Bertino, Blood, 86 (1995) 677.
[11] K.J. Ellis and J.F. Morrison, Methods Enzymol., 87 (1982) 405.
[12] M. Bradford, Anal. Biochem., 21 (1976) 178.
[13] X. Huang, W.F. Coleman and R.N. Zare, J. Chromatogr., 480 (1989) 95.
[14] K.D. Altria, Chromatographia, 35 (1993) 177.
[15] V.M. Whitehead, D.S. Rosenblatt, M.J. Vuchich, J.J. Shuster, A. Witte and D. Beaulieu, Blood, 76 (1990) 44.
[16] J.T. Lin, W.P. Tong, T.M. Trippett, D. Niedzwiecki, Y. Tao, C. Tan, P. Steinherz, B.I. Schweitzer and J.R. Bertino, Leukemia Res., 15 (1991) 1191.
[17] E. Göker, J. Lin, T.M. Trippett, Y. Elisseyeff, W. Tong, D. Niedzwiecki, C. Tan, P. Steinherz, B. Schweitzer and J.R. Bertino, Leukemia, 7 (1993) 1000.