Synthesis, Retention, and Biological Activity of Methotrexate Polyglutamates in Cultured Human Breast Cancer Cells

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ABSTRACT To determine the pharmacologic importance of methotrexate (MTX) polyglutamates, we examined the formation, retention, and effect of these metabolites in cultured human breast cancer cells. Two cell lines (MCF-7 and ZR-75-B) converted the drug to γ-polyglutamate derivatives in a dose- and time-dependent reaction. After 24-h incubations with 2 μM MTX, polyglutamates of two to five amino acids in length accounted for 55.4% (51.9 nmol/g) of intracellular drug in the MCF-7 cells and 87.6% (62.4 nmol/g) of drug in ZR-75-B cells. In contrast, MDA-231 cells showed lesser accumulation of MTX, and only 32% (4.06 nmol/g) of the intracellular drug was in the form of polyglutamates, a difference that could only partially be explained by decreased ability of these cells to take up free drug from the medium. When MCF-7 and ZR-75-B cells containing polyglutamates were transferred to drug-free medium for 24 h, 22 and 51% of the total intracellular drug were, respectively, retained in each cell line. The loss of intracellular drug was primarily accounted for by disappearance of parent compound and polyglutamates containing 1–3 additional glutamyl residues. The rates of disappearance from cells decreased with increasing glutamyl chain length. All of the 4-NH_2-10-CH_3-$PteGlu_5$ and 47 and 38% of the 4-NH_2-10-CH_3-$PteGlu_4$ remained in the MCF-7 and ZR-75-B cells, respectively, and could be identified in the cytosol after 24 h in drug-free medium. The retention of MTX polyglutamates in these two cell lines in excess of dihydrofolate reductase binding capacity led to prolonged inhibition of thymidylate synthesis and loss of cell viability after removal of extracellular MTX. After 24-h incubation with 2 μM MTX and an additional 24 h in drug-free medium, [^{3}H]deoxyuridine incorporation was still inhibited to 30% of control in the MCF-7 cells and 34.7% of control in ZR-75-B cells; this persistent inhibition was associated with a 30% reduction in cell numbers in each cell line during the 24-h period in drug-free medium. In contrast, [^{3}H]deoxyuridine incorporation and cell growth quickly recovered to normal in the MDA-231 cells following removal of 2 μM MTX from the medium after a 24-h incubation. Prolonged inhibition of both thymidylate synthesis and cell growth was observed in this cell line in drug-free medium only after a 24-h incubation with 10 μM MTX, a condition that leads to the synthesis of 11.3 nmol/g of MTX polyglutamates.

These studies demonstrate that polyglutamate formation allows a prolonged retention of drug in a nonefluxable form and prolonged inhibition of both thymidylate synthesis and cell growth following removal of extracellular drug.

INTRODUCTION

Methotrexate (2,4-diamino,N^{10}-methylpteroyl glutamic acid; MTX),[1] a potent inhibitor of dihydrofolate reductase (DHFR), has important antitumor action against a broad spectrum of malignancies. The parent compound has been the subject of intensive investigation with respect to its active transport across cell membranes, its tight binding to reductase, its conversion to inactive metabolites, and its elimination by

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Received for publication 23 November 1981 and in revised form 2 April 1982.

[1] *Abbreviations used in this paper*: DHFR, dihydrofolate reductase; dI, deoxyinosine; dT, thymidine; dU, deoxyuridine; FCS, fetal calf serum; HPLC, high pressure liquid chromatography; IMEM, improved Minimal Essential Medium; MTX, methotrexate; MTXPG, polyglutamate derivatives of MTX; PBS, phosphate-buffered saline.

urinary excretion and metabolism (1). The cytotoxic effects of this agent appear to depend on the maintenance of intracellular drug concentrations in excess of its target enzyme, since its biochemical effects are rapidly reversed by washout of the free drug from the intracellular space (2). The reversible nature of reductase inhibition is believed to result from competition of drug and the expanded pool of physiologic substrate, dihydrofolate, for binding sites on the enzyme.

Like the physiological folate cofactors (3), MTX is metabolized to poly-γ-glutamyl derivatives by many tissues: in vivo by human erythrocytes (4), liver (5), and by murine tissues and tumors (6–9), and in vitro by human fibroblasts (10), bone marrow aspirates (11), rat hepatocytes and hepatoma cells (12, 13), and Ehrlich ascites tumor cells (14). In previous studies, we have shown that after 24 h of incubation with 2 μM MTX, human breast cancer cells contained predominantly polyglutamate derivatives of methotrexate (MTXPG) varying in length from one to four additional glutamates, both free in the cytoplasm and bound to DHFR (15, 16). The pharmacologic role of MTXPG is uncertain, although evidence has been reported that these compounds are preferentially retained by cells after removal of extracellular drug. Preferential retention of MTXPG has been described in studies of human fibroblasts (17), rat hepatoma cells (13), and Ehrlich ascites tumor cells (14); this retention by cells in culture has been associated with a prolongation of the drug's biochemical blockade of thymidylate synthesis (13, 17) and its cytotoxic effects (13). However, there does not exist general agreement regarding the capacity of polyglutamates to exit from cells. Poser et al. found that the MTXPG were not retained in vitro (8) or in vivo (9) by L1210 cells. The behavior of these metabolites may thus vary among different tissues and tumors of different species.

In the present studies, we have examined the formation, retention, and pharmacologic effects of MTXPG in human breast cancer cell lines, using a highly specific high pressure liquid chromatography (HPLC) analytic system that allows precise identification of chain length of the derivatives (15). These studies indicate that considerable differences exist in the ability of human breast cancer cell lines to form these derivatives, that each of the polyglutamates has different retention characteristics (the longer derivatives remaining free in the cytosol for at least 24 h after removal of extracellular drug), and that polyglutamates significantly prolong the cytotoxic action of this drug.

METHODS

Chemicals. [3',5',9-^{3}H]MTX (20 Ci/mmol sp act) and [^{14}C]carboxylic acid inulin (13.5 mCi/mmol sp act) were purchased from Amersham Corp. (Arlington Heights, IL). [^{3}H]MTX was further purified by DEAE-cellulose chromatography with elution along a linear gradient of 0.1–0.4 NH_4HCO_3, pH 8.3 (18). Unlabeled MTX was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD) and was purified by the same procedure. Purified synthetic 4-NH_2-10-CH_3-$PteGlu_{2-4}$ were provided by Dr. John Montgomery (Southern Research Institute, Birmingham, AL) and Dr. C. M. Baugh (Department of Biochemistry, University of South Alabama, Mobile, AL) (19). [6-^{3}H]Thymidine (21.5 Ci/mmol sp act), [6-^{3}H]-deoxyuridine (23.3 Ci/mmol sp act), [^{3}H]H_2O (1 mCi/ml sp act) and Aquassure liquid scintillation counting fluid were purchased from the New England Nuclear Corp. (Boston, MA), and L-glutamine from Flow Laboratories, Inc. (Camden, CT). Acetonitrile (CH_3CN) and tetrabutyl ammonium phosphate (PicA) were obtained from Waters Associates (Milford, MA) and DEAE-Sephacell from Pharmacia (Uppsala, Sweden). Versilube F50 silicone fluid was purchased from General Electric Co. (Waterford, NY). All other chemicals were of reagent grade and purchased either from Fisher Scientific Co. (Pittsburgh, PA) or Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was obtained from Biofluids, Inc. (Rockville, MD) and dialyzed as follows: 100 ml of FCS was put in Spectrapor (3787-D22) tubing (A. H. Thomas Co., Philadelphia, PA) and dialyzed against 8 liters of 0.9 g % NaCl containing Ag-1-X8 resin (Bio-Rad Laboratories, Richmond, CA) at 4°C. The completeness of the dialysis was determined by the disappearance of [^{3}H]-thymidine added to the FCS before dialysis.

Propagation of cells in culture. MCF-7, MDA-231, and ZR-75-B cells (passage 59 and 60), three lines of human breast cancer cells in continuous monolayer culture, were provided by Dr. Marc Lippman (National Cancer Institute). The characteristics of the MCF-7 and MDA-231 cell lines have been previously described (20, 21). The ZR-75-B cell line is a cloned line derived from the ZR-75-1 cell line (22) and has the same hormonal receptors and responsiveness as the parent cell line. The cells were grown in improved Minimal Essential Medium (IMEM; National Institutes of Health Media Unit, Bethesda, MD) supplemented with 10% FCS, 4 mM L-glutamine, penicillin at 124 μg/ml, and streptomycin 270 μg/ml under 5% CO_2 at 37°C. For experiments examining intracellular MTXPG formation, the cells were first incubated in 75-cm^2 plastic flasks (Costar, Data Packaging, Cambridge, MA) for various periods in folate-free IMEM containing 0.2, 2, or 10 μM [^{3}H]MTX with 2 mM L-glutamine, 10 μM thymidine (dT), and 10 μM deoxyinosine (dI). This medium allowed cell growth to proceed unperturbed in the presence of MTX. At the end of incubation, cell samples were obtained for analysis of intracellular content of MTX and metabolites as described below. To determine cell numbers, the cells were detached from the flask surface by 0.05% trypsin and 0.02% EDTA at 37°C for 10 min and counted in a model B Coulter counter (Coulter Electronics, Hialeah, FL).

Assay of intracellular MTXPG. The intracellular content of MTX and its poly-γ-glutamyl metabolites was determined as follows: at the end of an incubation period with MTX, the medium was aspirated and the cells were washed with ice-cold phosphate-buffered saline (PBS). The cells were scraped off the flask surface with a rubber policeman and lysed by adding 4.5 ml of ice-cold water. The cell lysate was quickly transferred to a test tube containing 0.5 ml of 100% TCA so that a final concentration of 10% TCA was achieved. Cellular debris was pelleted by centrifugation at 10,000 *g* for 15 min and the cell extract was injected into a Sep-Pak C_{18} cartridge (Waters Associates), which had been prepared by

prior injection with 2 ml of 100% CH_3CN followed by 5 ml of water. The cartridge was then washed by injecting 5 ml of water, following which MTX and its metabolites were eluted with 2 ml of CH_3CN. The sample was evaporated to dryness under N_2 and resuspended in the initial HPLC mobile phase. Of the radioactivity present in the TCA extract, 71±10% was recovered in the resuspended sample. The unrecovered drug was not adsorbed on the cartridge and was lost during sample injection and cartridge washing. The recovery rates of authentic MTX and 4-NH_2-10-CH_3-PteGlu_{2-4} were tested individually using the Sep-Pak procedure and determined to be the same for each compound. The total amount of intracellular drug was determined in nanomoles per gram as calculated from the radioactivity in the TCA supernatant, the specific activity of [^{3}H]MTX used, and the amount of protein in the TCA precipitate, as measured by Lowry protein assay (23).

MTX and MTXPG were separated using a recently developed HPLC assay (16), modified to give a more rapid separation. The mobile phase was prepared by a model 660 solvent programmer (Waters Associates) mixing the effluents from two model 6000A pumps (Waters Associates). Pump's A solvent was KH_2PO_4 10 mM with PicA 5 mM at pH 5.5, while pump B contained 100% CH_3CN. Sample fractions (10–50 μl) containing ~2,000 dpm were injected onto a Radial-Pak C_8 cartridge (Waters Associates) in an RCM-100 Radial Compression Module (Waters Associates) and eluted at 2 ml/min along gradients of 21–27% CH_3CN and 3.95–3.65 mM PicA for 15 min followed by 27% CH_3CN and 3.65 mM PicA for the last 15 min of the separation. The retention times of authentic 4-NH_2-10-CH_3PteGlu_{1-4} were determined by monitoring UV absorbance at 313 nm. 1-min fractions were collected directly into scintillation vials using an LKB 2112 Redirac fraction collector (LKB-Produkter-AB, Bromma, Sweden) and assayed for radioactivity by liquid scintillation counting.

Characterization of DHFR-bound and -free intracellular MTX. To separate drug bound to DHFR from drug free in the cytosol, MCF-7 and ZR-75-B cells were incubated with 2 μM [^{3}H]MTX for 24 h as described previously. After the incubation, the cells were washed once with ice-cold PBS and placed in drug- and folate-free IMEM for 24 h. The medium was changed after 1 and 6 h of efflux. At the end of the incubation and after the period in drug-free medium, the cells were washed with ice-cold PBS, scraped off the flask surface with a rubber policeman, and lysed by adding 1 ml of KH_2PO_4 0.15 M, pH 6.2. Cytosol was obtained by centrifugation at 100,000 *g* for 30 min at 4°C in a Beckman L5-50 ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) and applied to a 0.8 × 2.5 cm DEAE-Sephacell minicolumn equilibrated with 2 column vol of 0.15 M KH_2PO_4, pH 6.2, at 4°C. Protein-bound drug was first eluted with 4 ml of the same buffer, following which unbound drug was recovered with 5 ml of 1 M NH_4HCO_3. This procedure was standardized using authentic [^{3}H]MTX and [^{3}H]-4-NH_2-10-CH_3-PteGlu_{2-5} isolated and purified from ZR-75-B cells after a 24-h incubation with 2 μM [^{3}H]MTX. Since we have previously shown that MTX and MTXPG are not associated with proteins other than DHFR in MCF-7 cell extracts (15), we determined the elution patterns of DHFR-bound MTX and MTGPG on the DEAE-Sephacell minicolumn by the following procedure: 60,000 dpm (100 λ) of tritiated drug and 0.24 μmol of NADPH were added to a 1-ml MCF-7 cytosol preparation and incubated at room temperature for 15 min. The unbound drug was then removed by adding 200 μl of a charcoal slurry (24), which was spun down before applying the supernatant to the column. For all the compounds tested, virtually all DHFR-bound drug eluted in the 0.15 M KH_2PO_4 wash. To determine the elution patterns of unbound drug, MTX (final concentration 0.1 mM) was first added with 0.24 μmol NADPH to a 1-ml MCF-7 cytosol preparation to saturate all DHFR binding sites. [^{3}H]MTX and [^{3}H]4-NH_2-10-CH_3-PteGlu_{2-5} were then added to the cytosol that was applied to the column. Over 96% of the unbound MTX and >99% of the unbound 4-NH_2-10-CH_3-PteGlu_{2-5} eluted in the NH_4HCO_3 wash.

Determination of cell water content. The volume of intracellular water per gram of cells was determined in each cell line using a method modified from Wohlhueter et al. (25). Cell suspensions of 10^6 cells/ml were prepared by detaching cells from the flask surfaces with 3 ml of 0.05% trypsin and 0.02% EDTA at 37°C for 5 min and quickly adding 7 ml of IMEM with 10% FCS to neutralize the trypsin. [^{3}H]H_2O or [^{14}C]carboxylic acid inulin was then added to the cell suspension to obtain a final concentration of 2,000 dpm/μl. 1 ml of this suspension was layered on top of 0.6 ml of silicone fluid in a 1.6-ml conical tube (Sarstedt, West Germany) and the cells were spun through the silicone and pelleted at 12,000 *g* for 1 min in an Eppendorf 5413 centrifuge (Brinkmann Instruments, Inc., Westbury, NY). The cell pellet was then dissolved in 1 ml of 1 N NaOH, and half of this was assayed for radioactivity, while a Lowry protein assay (23) was performed on the other half. The total [^{3}H]H_2O and [^{14}C]inulin volumes (milliliters per gram) were determined by dividing the ^{3}H or ^{14}C disintegrations per minute per gram of protein in the cell pellets by the disintegrations per minute per milliliter in the initial cell suspension. The intracellular [^{3}H]H_2O volume was calculated for each cell line by substraction of the extracellular (inulin) volume from the total [^{3}H]H_2O volume.

Assay for deoxyuridine (dU) incorporation. [^{3}H]dU incorporation was measured in the human breast cancer cells at the end of various incubations with MTX and again after resuspending the cells for 1, 6, 12, and 24 h in drug-free medium. The cells were first incubated in 9.62 cm^2 wells (Flow Laboratories, Inc.) for various periods in 2 ml of folate-free IMEM containing varying concentrations of MTX with 2 mM L-glutamine, 10 μM dT, and 100 μM dI. This medium prevented cytotoxicity in the presence of MTX. At the end of incubation, the medium was aspirated from all the wells, the cells were washed once with 2 ml ice-cold PBS and placed in 2 ml of IMEM containing 10% dialyzed FCS with 1 U insulin/100 ml and 100 μM dI. [^{3}H]dU incorporation was then measured at this point by exposing the cells to 1 μM [^{3}H]dU for 45 min at 37°C, following which the medium was aspirated and the cellular macromolecules precipitated *in situ* with 10% TCA. The precipitates were washed two times with 2 ml PBS, solubilized in 1 ml 1 M NaOH, and half of this final sample assayed for radioactivity, while a Lowry protein assay (23) was performed on the other half. Results were expressed in disintegrations per minute per milligram protein as a percentage of the values obtained in control cells growing in drug-free medium. The incorporation of 1 μM [^{3}H]dT was also determined under the same conditions to assess cell viability. The drug-free efflux medium was replaced by fresh medium in all remaining wells before [^{3}H]dU and [^{3}H]dT incorporation measurements at 1, 6, 12, and 24 h after the end of incubation.

RESULTS

MTXPG formation in human breast cancer cells. We first examined the formation of MTXPG in MCF-7 cells incubated continuously with 2 μM [^{3}H]MTX for

1–24 h in folate-free IMEM containing 10 μM dT and dI to prevent cytotoxicity. As shown in Fig. 1, after a 1-h incubation, 92% of the intracellular radioactivity consisted of an early peak that cochromatographed with authentic MTX, while a small second peak constituting 8% of the total intracellular drug cochromatographed with authentic 4-NH_2-10-CH_3-$PteGlu_2$. After 6 h, two additional peaks accounting, respectively, for 15.3 and 4.6% of the total disintegrations per minute appeared and cochromatographed with authentic 4-NH_2-10-CH_3-$PteGlu_{3\&4}$, while the formation of 4-NH_2-10-CH_3-$PteGlu_2$ increased to 16.9% of total intracellular drug. After 12 h, a fifth peak appeared that represented 3.5% of the total disintegrations per minute, and eluted on HPLC with a retention time consistent with 4-NH_2-10-CH_3-$PteGlu_5$ (16). At the end of the 24-h incubations, 4-NH_2-10-CH_3-$PteGlu_{3\&4}$ had further increased to 14.9 and 6.6%, respectively, of the total disintegrations per minute and the MTXPG accounted for 54.7% of the total intracellular drug. Their formation in the period from 1 to 24 h was associated with an increase in the total amount of intracellular drug from 27.5 nmol/g at the end of the 1-h incubation to 63.5 nmol/g at the end of incubation. Since the intracellular MTX content only increased from 25.3 to 28.7 nmol/g in the last 23 h of incubation, 91% of the further accumulation of intracellular drug after 1 h was accounted for by the formation of MTXPG.

We next examined the concentration-dependent formation of MTXPG in the MDA-231, MCF-7, and ZR-75-B cell lines after 24-h incubation with either 0.2, 2, or 10 μM [^{3}H]MTX. Representative experiments are illustrated in Fig. 2; the ZR-75-B cells consistently synthesized relatively more 4-NH_2-10-CH_3-$PteGlu_{4\&5}$ at 2 and 10 μM MTX than the MCF-7 cells. The MDA-231 cells, however, accumulated less total drug and lesser quantities of MTXPG at all concentrations than the other two cell lines. A comparison of the absolute amounts of MTX and MTXPG formed in the MCF-7 and ZR-75-B cells after 24-h incubations with 2 μM [^{3}H]MTX is shown in Table I. A greater percentage of total intracellular drug was in the form of MTXPG in the ZR-75-B cells (87.6 vs. 55.4%) and this line formed

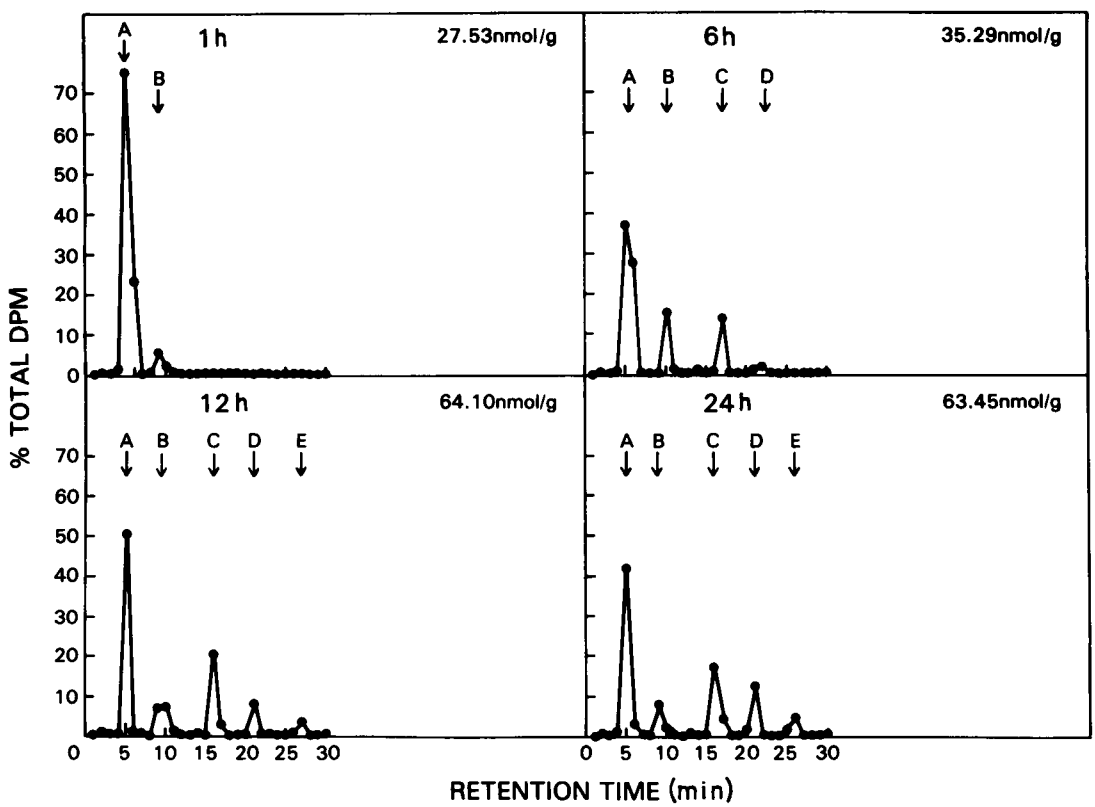

FIGURE 1 Time course of MTXPG formation in MCF-7 cells. After 1-, 6-, 12-, and 24-h incubations with 2 μM [^{3}H]MTX, cell extracts were chromatographed by HPLC. Peaks A to E represent, respectively, 4-NH_2-10-CH_3-$PteGlu_1$ (MTX) to 4-NH_2-10-CH_3-$PteGlu_5$. The total amount of intracellular drug in nanomoles per gram of cell protein is given at each time point.

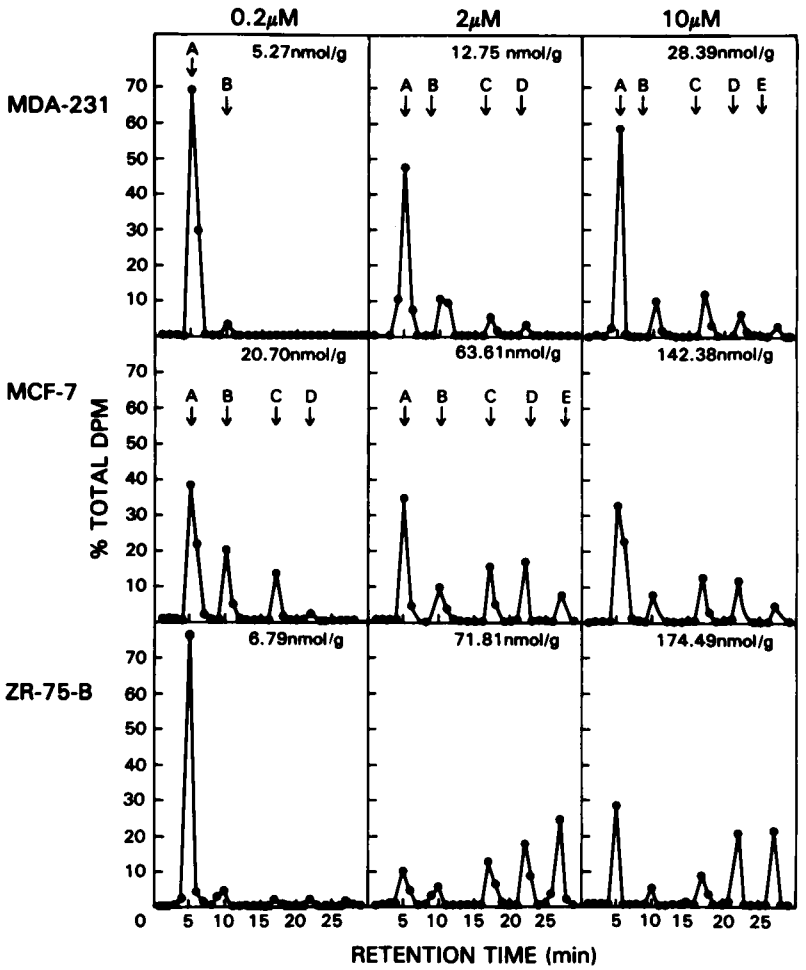

FIGURE 2 Dose-response of MTXPG formation in human breast cancer cells. 24-h incubations with either 0.2, 2, or 10 μM [^{3}H]MTX were done in the MDA-231, MCF-7, and ZR-75-B cells, following which cell extracts were chromatographed by HPLC. Peaks A to E represent, respectively, 4-NH_2-10-CH_3-PteGlu$_1$ (MTX) to 4-NH_2-10-CH_3-Pte-Glu$_5$. The total amount of intracellular drug in nanomoles per gram of cell protein is given at each time point.

2.5 times as much 4-NH_2-10-CH_3-PteGlu$_5$ compared with the MCF-7 cell line.

Uptake and binding capacity for MTX. To determine if the different rates of MTXPG formation in the three cell lines were due to different levels of substrate (free intracellular MTX) available to the enzyme folyl polyglutamate synthetase, we measured in each cell line the two factors that determine free intracellular levels of MTX: drug uptake and intracellular binding capacity for MTX. Drug uptake was assessed by measuring intracellular MTX levels during 1-h incubations with 2 μM MTX, conditions which were not associated with significant MTXPG synthesis in any cell line. In the three cell lines, a plateau of intracellular MTX was reached after 30 min of incubation. As shown in Table II, uptake plateau levels were 13.2 and 13.3 nmol/g, respectively, in the MCF-7 and ZR-75-B cells, while they were only 4.11 in the MDA-231 cells. To confirm that these levels were sufficient to fully saturate the cells' intracellular binding capacity for MTX, the latter was determined by measuring intracellular drug concentrations during a 1-h period in drug-free medium following the 1-h incubation with drug. In each cell line, a fall in intracellular drug level to a plateau representing the intracellular binding capacity was observed after 30 min in drug-free medium. As seen in Table II, the efflux plateau levels were similar in the MCF-7 and ZR-75-B cells (3.73 and 5.49 nmol/g, respectively), while the level of 0.82 nmol/g in the MDA-231 cells was much lower than in the two other cell lines. Direct measurement of DHFR activity in each cell line confirmed that higher enzyme levels were

TABLE I

Distribution of Intracellular MTXPG in MCF-7 and ZR-75-B Cells after 24-h Incubations with 2 μM MTX [24(0)] and after an Additional 24 h in Drug-free Medium [24(24)]

	MCF-7		ZR-75-B	
	24(0)	24(24)	24(0)	24(24)
Total intracellular drug (*nmol/g*)	95.4±9.0	20.9±1.8	71.39±5.53	36.06±0.64
Amounts of intracellular 4-NH_2-10-CH_3-$PteGlu_n$				
$n = 1$	42.7±7.1	0.6±0.1	8.9±2.5	0.4±0.1
$n = 2$	10.3±0.6	0.4±0.2	4.6±0.5	0.4±0.2
$n = 3$	17.6±1.1	3.2±0.5	12.2±1.6	1.6±0.4
$n = 4$	14.2±1.5	6.7±0.7	20.9±1.1	8.0±0.7
$n = 5$	9.8±0.9	9.9±0.5	24.7±1.3	25.6±0.8

After 24-h incubations with 2 μM [^{3}H]MTX and after another 24 h in drug-free medium, cell extracts of MCF-7 and ZR-75-B cells were assayed by HPLC for MTXPG. The mean total amount of intracellular drug (nanomoles per gram) and the mean amounts of 4-NH_2-10-CH_3-$PteGlu_{n=1-5}$ are shown with standard deviations for triplicate experiments.

present in the MCF-7 and ZR-75-B cells as compared with the MDA-231 cells (Table II). Consequently, the three cell lines could transport intracellularly enough MTX to saturate their drug-binding capacity and accumulate free intracellular drug at 2 μM MTX. The two cell lines that synthesize large amounts of MTXPG over a 24-h period at this concentration—51.9 nmol/g in the MCF-7 and 62.4 nmol/g in the ZR-75-B—had similar levels of free intracellular drug (9.44 and 7.76 nmol/g, respectively), while the MDA-231 cells, which form only 4.06 nmol/g MTXPG at 2 μM MTX, had lower but detectable levels of free drug (3.3 nmol/g). Furthermore, the MDA-231 cells could accumulate 47.2±0.95 nmol/g of free MTX after a 1-h incubation with 10 μM MTX yet only form 11.3 nmol/g of MTXPG after a 24-h incubation with the same drug concentration. Because the intracellular [^{3}H]H_2O volumes were similar in the three cell lines (Table III), the lower intracellular drug levels in the MDA-231 cells measured after the incubation with 2 μM MTX could not be explained by relative differences in cell water content between the cell lines. Thus, the marked reduction of MTXPG synthesis in the MDA-231 cells can only partially be explained by an insufficient substrate level, and is also probably the result of a deficient activity of the enyzme folyl polyglutamate synthetase.

TABLE II

MTX Transport Characteristics and Enzyme Activity in Human Breast Cancer Cells

Cell line	Uptake plateau level	Efflux plateau level	Free intracellular drug	Enzyme activity
		nmol/g		*μmol/h/mg*
MDA-231	4.11±1.18	0.82±0.19	3.3±1.17	0.033
MCF-7	13.2±1.02	3.73±0.37	9.44±0.69	0.097
ZR-75-B	13.3±4.3	5.49±2.88	7.76±1.82	0.078

The intracellular amounts of MTX (nanomoles per gram) were determined in MDA-231, MCF-7, and ZR-75-B cells after 30-, 45-, and 60-min incubations with 2-μM [^{3}H]MTX as described by Schilsky et al. (26), and the mean of these three determinations represents the uptake plateau. Intracellular amounts were also determined after drug efflux in drug-free medium following a 60-min incubation with 2 μM MTX. At the end of drug incubation, drug uptake was stopped by the rapid addition of 5 vol of PBS at 4°C. The cells were washed in 2 vol PBS before being placed in folate-free IMEM. Drug amounts were determined after 30, 45, and 60 min in drug-free medium and the mean of these three determinations represents the efflux plateau. Free intracellular drug level was calculated by subtracting the efflux plateau from the uptake plateau level. The mean results and standard deviations from triplicate experiments are shown. DHFR activity was determined spectrophotometrically in each cell line as previously described (27).

Retention of MTXPG by human breast cancer cells. The ability of the MCF-7 and ZR-75-B cells to retain MTX and MTXPG in drug-free medium was next investigated. After 24-h incubations in 2 μM [^{3}H]MTX, the amounts of MTX and MTXPG were determined, and cells were transferred to drug-free medium (IMEM). The results are detailed in Table I. In both

TABLE III
Cell Water Content of the Human Breast Cancer Cells

Cell line	Total [^{3}H]H_2O (ml) / Cell pellet weight (g)	Extracellular [^{14}C]inulin (ml) / Cell pellet weight (g)	Intracellular [^{3}H]H_2O (ml) / Cell pellet weight (g)
MDA-231	9.96±0.22	1.92±0.21	8.04
MCF-7	9.93±0.83	1.01±0.18	8.92
ZR-75-B	9.45±0.59	1.65±0.18	7.80

The total [^{3}H]H_2O volume (milliliters per gram of cell protein) and the [^{14}C]inulin extracellular volume (milliliters per gram) were determined in each cell line. The mean result and standard deviation of four experiments are shown. The intracellular [^{3}H]H_2O volume was calculated by subtracting the [^{14}C]inulin space from the total [^{3}H]H_2O volume.

cell lines, drug retention directly correlated with the number of glutamyl residues. During the 24-h efflux period, the MCF-7 cells retained 22% of total drug and ZR-75-B retained 51% of the drug present in the cells at the end of the initial incubation period. In the MCF cells, 60% of drug efflux occurred in the 1st h in drug-free medium and was almost completely accounted for (97%) by the loss of parent drug. By 6 h in drug-free medium in both cell lines, >90% of the 4-NH_2-10-CH_3-$PteGlu_2$ had left the cells. In the last 18 h in drug-free medium, 81.8 and 86.9% of the 4-NH_2-10-CH_3-$PteGlu_3$ and 42.8 and 51.7% of the 4-NH_2-10-CH_3-$PteGlu_4$ were lost by the MCF-7 and ZR-75-B cells, respectively, while 4-NH_2-10-CH_3-$PteGlu_5$ was completely retained. The greater drug retention by the ZR-75-B cells was explained by the greater amount of 4-NH_2-10-CH_3-$PteGlu_5$ present in these cells before the efflux period.

Preferential retention of 4-NH_2-10-CH_3-$PteGlu_{4\&5}$ in the two cell lines could be due either to preferential binding of these derivatives to DHFR or their selective retention by the cell membrane when free in the cytosol. Two lines of evidence suggest that the second hypothesis is correct. First, after the 24-h efflux period in both cell lines, the total amount of intracellular drug exceeded each cell's binding capacity. Tables I and II show that the intracellular drug level in the MCF-7 cells after 24-h efflux was 20.9 nmol/g, which exceeded the MTX binding capacity of 3.73 nmol/g. In the ZR-75-B cells, the drug level was 36.06 nmol/g compared with a binding capacity of 5.49 nmol/g. Analysis of the unbound fractions of intracellular drug following a 24-h incubation with 2 μM MTX and after 24 h in drug-free medium confirmed the retention of free intracellular MTXPG. After the period in drug-free medium, 76.1 and 64.0% of the 4-NH_2-10-CH_3-$PteGlu_5$ found free in the MCF-7 and ZR-75-B cell cytosol, respectively, at the end of the drug incubation was still present in the unbound fraction, while only 26.1 and 32.5% of 4-NH_2-10-CH_3-$PteGlu_4$, 9.5 and 17.8% of 4-NH_2-10-CH_3-$PteGlu_3$, and <5% of MTX and 4-NH_2-10-CH_3-$PteGlu_2$ had been retained unbound in the MCF-7 and ZR-75-B cells. Thus, 4-NH_2-10-CH_3-$PteGlu_{4\&5}$ remained free in the cytosol for at least 24 h after the removal of parent drug from the extracellular medium.

[^{3}H]dU incorporation studies. To determine the effects of MTXPG retention on thymidylate synthesis, we monitored the folate-dependent incorporation of [^{3}H]dU into DNA in the three human breast cancer cell lines at the end of various MTX incubations. The chosen conditions were those associated with little or maximal MTXPG synthesis (Table IV). In all instances, drug incubations associated with significant MTXPG formation led to marked inhibition of [^{3}H]dU incorporation at the end of incubation with drug, and this inhibition persisted even after 24 h in drug-free medium. MTX incubations leading to little MTXPG synthesis were associated with only partial inhibition of [^{3}H]dU incorporation at the end of the incubation period and in all instances [^{3}H]dU incorporation returned to normal levels within periods of 1–6 h in drug-free medium. In the three cell lines, [^{3}H]dT incorporation was the same in the drug-treated and control cells when 100 μM dI was added to the efflux medium. This confirmed that the prolonged inhibition of [^{3}H]dU incorporation was due to continued DHFR inhibition and not to loss of cell viability.

Growth inhibition by retained MTXPG. We next determined if the prolonged inhibition of [^{3}H]dU incorporation in drug-free medium after initial incubations with MTX was associated with impaired growth of the breast cancer cells. There were two phases of 24-h cell incubations in these experiments. In the first phase, the cells were incubated with MTX in the presence of 10 μM dT and 10 μM dI, a medium that supported cell growth despite the presence of drug. After this incubation, cells were next placed in a drug-free medium that supported cell growth under control conditions, but did not contain sufficient pu-

TABLE IV
[^{3}H]dU Incorporation Studies

		MTXPG formation	[^{3}H]-dU incorporation	
			End of incubation	24 h in drug-free medium
			% control	
MDA-231	A: 2 μM MTX for 24 h	−	58.9±3.9	106.6±11.7
	B: 10 μM MTX for 24 h	+	7.9±7.5	28.7±15.6
MCF-7	A: 2 μM MTX for 1 h	−	31.3±13.1	92.1±5.2
	B: 2 μM MTX for 24 h	+	3.4±1.2	30.2±3.9
ZR-75-B	A: 0.2 μM MTX for 24 h	−	55.9±6.6	102.0±25.0
	B: 2 μM MTX for 24 h	+	2.9±1.3	34.7±20.6

[^{3}H]dU incorporation was measured in the human breast cancer cell lines at the end of MTX incubations leading to little MTXPG synthesis (condition A), or significant MTXPG synthesis (condition B). In each instance [^{3}H]dU incorporation was measured again after 24 h in drug-free medium. Results were determined in disintegrations per minute per milligram of cell protein and reported as a percentage of control values. Each point represents the mean results with standard errors for three duplicate experiments in the MCF-7 cells and two duplicate experiments in the MDA-231 and ZR-75-B cells.

rines or pyrimidines to rescue the cells from MTX antifolate effects (IMEM with 10% dialyzed FCS and 1 U of insulin/100 ml). Cell growth proceeded unperturbed during the drug exposure period. Consequently, any effect on cell growth noted in the subsequent 24 h (the second phase) in drug- and rescue-free medium would likely be secondary to persistent inhibition of DHFR by retained MTXPG. As shown in Table V, growth of MCF-7 and ZR-75-B cells in the second incubation period was inhibited to 75.2 and 70.3% of control, respectively, following a first-phase incubation with 2 μM MTX and to 68.9 and 65% of control following first phase incubation with 10 μM MTX. Using a two-way analysis of variance, these re-

TABLE V
MTX Inhibition of Cell Growth

Cell line	MTX concentration during initial incubation	MTXPG formation	Cell number	
			After 24-h incubation with MTX	After 24-h in drug-free medium
	μM		*% control*	
MDA-231	2	−	97.2	98.7
	10	+	93.4	70.5*
MCF-7	0.2	−	95.5	96.7
	2.0	+	97.4	75.2*
	10.0	+	87.8	65*
ZR-75-B	0.2	−	115.5	94.5
	2.0	+	100.6	70.3*
	10.0	+	98.1	68.9*

Cells were incubated for 24 h in medium containing 10 μM dT and 10 μM dI, and the indicated concentration of MTX. Cells were then washed once with PBS at 4°C and placed in drug-free medium containing 10% dialyzed FCS and insulin. Cell counts were performed at time zero (control), after 24 h with drug, and after 24 h in drug-free medium. Each point represents the mean result of two experiments, each run in duplicate. Results were analyzed using a two-way analysis of variance, comparing control and postincubation cell counts.
* $P < 0.01$.

sults were significantly different from control cell growth at the $P < 0.01$ level. Exposure to 0.2 μM MTX in the first phase did not result in any significant growth delay in the second phase in either cell line. In the MDA-231 cells, second phase cell growth was unaffected by initial incubation with 2 μM MTX, while the cell number decreased to 70.5% of control in the second phase after initial incubation with 10 μM MTX, a change significant at the $P < 0.01$ level. Cell growth inhibition could be reversed in all three cell lines by addition of 10 μM dT and 100 μM dI to the drug-free medium. Thus, in each case the intracellular accumulation of MTXPG during an initial incubation period was associated with a persistent inhibition of cell growth evident even after 24 h in drug-free medium.

DISCUSSION

The present experiments have described the time- and concentration-dependent formation of MTXPG of two to five glutamyl residues in length in three human breast cancer cell lines. Clear differences in the rates of MTXPG formation and in the specific chain length of the products were observed among these cell lines under conditions of 24-h exposure to clinically relevant concentrations of MTX. While MDA-231 cells formed significant amounts of MTXPG only in the presence of 10 μM MTX, the two other cell lines, MCF-7 and ZR-75-B, synthesized sufficient MTXPG during 24-h incubations with 2 μM MTX to greatly increase the free intracellular drug concentration present at the end of a 1-h incubation, a crucial determinant of drug effect (2). In addition to quantitative differences in total MTXPG formation, the profile of MTXPG formation differed in the three cell lines, ZR-75-B cells consistently forming greater amounts of derivatives of 4 and 5 glutamyl chain length. In contrast, the Ehrlich ascites cells described by Fry et al. (14), as analyzed by HPLC, formed predominantly $4\text{-}NH_2\text{-}10\text{-}CH_3PteGlu_{3\&4}$ and much smaller amounts of Glu_5 after 4-h incubation with 5 μM MTX. Other cell lines studied, including L1210 murine leukemia (7–9) and rat hepatoma (13) formed predominantly the shorter chain length derivatives (1–3 glutamyl residues), although difference in drug concentrations, duration of exposure, and different methods of analysis (ion exchange or gel filtration) could account for these differences in comparison with the present work.

The profile of MTXPG formed appears to have an important effect on the ability of cells to retain drug after removal of MTX from the cell culture medium. We found a direct correlation between chain length and MTXPG retention, the Glu_5 derivative being detectable free in the cytosol 24 h after resuspension of cells in drug-free medium. Preferential retention of MTXPG has been observed in studies of human fibroblasts (17), rat hepatoma cells (13, 28), and Ehrlich ascites cells (14), although the specific relationship between glutamyl chain length and retention was not examined in these experiments. Other investigators have not confirmed the concept of preferential retention of MTXPG, either in vitro and in vivo in L1210 cells (8, 9) or in vivo in murine intestinal epithelium and bone marrow (9). However, the latter experiments (8, 9) examined tissues in which insignificant amounts of the higher MTXPG (4 and 5 glutamyl derivatives) were present, and the analyses were conducted by ion exchange chromatography. There is no doubt that the short chain length derivatives do exit the cell, but the rate of exit, as suggested by the present work, is clearly dependent on the number of glutamyl residues. From the work of Poser et al. (9), it appears likely that important normal murine tissue, specifically bone marrow and gastrointestinal epithelium, have lesser capacity to form the 4- and 5-glutamyl derivatives found in our breast cancer cell lines, a factor that may be responsible for selective drug action.

Our studies indicate that MTXPG formation and retention lead to a prolonged suppression of thymidylate synthesis and inhibition of cell growth. Incubation conditions that allowed MTXPG formation in excess of DHFR binding capacity led to a suppression of [^{3}H]dU incorporation into DNA for up to 24 h after removal of extracellular drug and rescue nucleosides. Others (13, 17, 28) have observed prolonged action of drug in association with MTXPG formation, although the present work represents the first documentation of the prolonged (24-h) retention of free intracellular drug in inhibited cells. However, it must be noted that a causal relationship between MTXPG retention and drug effect, while likely, has not been conclusively established.

Conditions that favor MTXPG formation in the breast cancer cells (higher drug level for longer period of time) might favor cell injury through mechanisms as yet undefined, despite the fact that the cells were protected by dT and dI during the drug incubation period. It would be desirable to show that mutants lacking folyl polyglutamate synthetase do not demonstrate prolonged inhibition of DNA synthesis and delayed cytotoxicity in drug-free medium.

The conversion of MTX to MTXPG must be taken into account in our current concepts of mechanisms of MTX action. MTXPG formation offers the possibility of explaining how a cell cycle-specific agent such as MTX exerts a cytotoxic effect on slow-dividing tumor cells such as human breast carcinoma. Prolonged retention of MTXPG and inhibition of DNA synthesis in the absence of free drug, which disappears from plasma within 24 h for most conventional dose regimens, could result from this process. The variability in the capacity of these three breast cancer cell lines

to form MTXPG further suggests that this process, as well as drug transport (29) and target enzyme concentration (30), may be an important determinant of *de novo* or acquired resistance to MTX.

ACKNOWLEDGMENTS

We would like to thank Dr. Richard Simon for doing the statistical analysis, Mr. Douglas Rennert for technical assistance, and Dr. Gregory Curt and Dr. Neil Clendeninn for their helpful suggestions and assistance.

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