Intracellular Pharmacokinetics of Methotrexate Polyglutamates in Human Breast Cancer Cells

SELECTIVE RETENTION AND LESS DISSOCIABLE BINDING OF 4-NH_2-10-CH_3-PTEROYLGLUTAMATE$_4$ AND 4-NH_2-10-CH_3-PTEROYLGLUTAMATE$_5$ TO DIHYDROFOLATE REDUCTASE

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ABSTRACT Methotrexate (MTX-Glu_1) exerts its antitumor effects through its potent inhibition of dihydrofolate reductase (DHFR), the enzyme responsible for maintaining the cellular pool of reduced folates. Since the drug-enzyme complex (bound drug) is slowly dissociable, an excess of drug (unbound or free drug) above that required to bind all enzyme sites is required in order to compete with substrate for sites made available by enzyme-drug dissociation. We have examined the role of the polyglutamyl metabolites of MTX-Glu_1 containing two to five glutamyl (MTX-Glu_{2-5}) groups in gamma peptide linkage in maintaining an intracellular pool of free drug and in forming slowly dissociable complexes with DHFR. During 24-h incubations of ZR-75-B human breast cancer cells with 2 μM MTX-Glu_1, we observed the progressive formation of derivatives with two to five glutamyl groups, which rapidly replaced the parent compound on enzyme binding sites and represented 85% of both unbound and bound intracellular drug at the end of incubation. When cells were then placed in drug-free medium, the rates of disappearance of drug and metabolites from the intracellular bound and free fractions decreased with increasing glutamyl chain length. Over 90% of both bound and free MTX-Glu_1 left the cells within 1 h, >90% of MTX-glu_2 left within 6 h, and >90% of MTX-Glu_3 left the bound and free fractions within 24 h. In contrast, free MTX-Glu_4 fell by only 63% and bound by only 23% affter 24 h, while free MTX-Glu_5 increased by 52% after 6 h in drug-free medium and bound MTX-Glu_5 increased threefold after 24 h, as it replaced the other forms of drug bound to DHFR. These results suggested a rapid dissociation of MTX-Glu_1 and -Glu_2 from the enzyme, and a slower dissociation of the longer chain length derivatives. This conclusion was confirmed by examining the rates at which [^{3}H]MTX-Glu_1 through -Glu_5 could be replaced on enzyme binding sites by a fivefold or greater excess of unlabeled MTX-Glu_1. Bound [^{3}H]MTX-Glu_1 and -Glu_2 had dissociation $t_{1/4}$ of 12 and 30 min, respectively, while -Glu_3, -Glu_4, and -Glu_5 had $t_{1/4}$ of 102, 108, and 120 min. These experiments demonstrated that the longer chain polyglutamates have prolonged intracellular retention and can be dissociated less readily than MTX-Glu_2 from DHFR, properties likely to make them more efficient DHFR inhibitors than the parent drug and of potential importance in extending the duration of drug action in tumor cells.

INTRODUCTION

Methotrexate (MTX-Glu_1)[1], the 2-4-diamino, 10-methyl analogue of folic acid, is thought to exert its antitumor effects through inhibition of dihydrofolate reductase (DHFR) (1). MTX-Glu_1 is a tight-binding but reversible inhibitor in the presence of NADPH, with slow but definite drug dissociation from the enzyme (2, 3). Consequently, MTX-Glu_1 must bind all enzyme catalytic sites (bound drug) and in addition must be pres-

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Received for publication 13 December 1982 and in revised form 27 April 1983.

[1] *Abbreviations used in this paper:* DHFR, dihydrofolate reductase; dI, deoxyinosine; dT, thymidine; FCS, fetal calf serum; FH_2, dihydrofolic acid; HPLC, high-pressure liquid chromatography; IMEM, improved minimal essential medium; MTX-Glu_1, methotrexate; MTXPG, MTX polyglutamates.

ent in excess of the fraction bound to the enzyme (unbound or free drug) in order to compete with the large pool of the physiologic substrate dihydrofolate waiting to replace bound drug as it dissociates from the enzyme (4). Like the physiological folate cofactors (5), MTX-Glu_1 is metabolized to poly-γ-glutamyl derivatives by many tissues, both in vivo and in vitro (6–14). Recent studies have shown that MTX polyglutamates (MTXPG) containing up to five glutamyl residues are synthesized in human breast cancer cell lines in vitro and that the metabolites with a total of four and five glutamyl residues (MTX-$Glu_{4\&5}$) are retained intracellularly and continue to inhibit enzymatic activity and cell growth at least 24 h after MTX removal from the extracellular fluid (14).

In the present work, we have further examined the kinetic profile of MTXPG in both the enzyme-bound and -free fractions in cultured human breast cancer cells. Using gel chromatography to separate bound from free drug, we have shown that each of the polyglutamate species becomes bound to DHFR in approximate proportion to its concentration in the free fraction, but only MTX-Glu_4 and Glu_5 are effectively retained intracellularly, both free in the cytosol and bound to DHFR, during long periods in drug-free medium. In addition, the latter compounds appear to be less readily dissociable from the enzyme than is MTX-Glu_1 and $_2$. Both characteristics, preferential intracellular retention and slow dissociation from DHFR, are likely to enhance the effectiveness of MTX as an inhibitor of DHFR.

METHODS

Chemicals. [3′,5′,9-^{3}H]MTX-Glu_1 (20 Ci/mmol sp act) was purchased from Amersham Corp. (Arlington Heights, IL) and further purified by DEAE-cellulose chromatography with elution along a linear gradient of 0.1–0.4 NH_4HCO_3 (15). Unlabeled MTX-Glu_1 was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD) and purified by the same procedure. L-Glutamine was obtained from Flow Laboratories, Inc. (McLean, VA); hydroxymethyl-aminomethane (Tris) from Boehringer Mannheim Biochemicals) Indianapolis, IN), DEAE-Sephacell from Pharmacia (Uppsala, Sweden), and dihydrofolic acid (FH_2) and 2-mercaptoethanol from Sigma Chemical Co. (St. Louis, MO). All other chemicals were of reagent grade and purchased from Fisher Scientific Co. (Pittsburgh, PA). Fetal calf serum (FCS) was obtained from Biofluids Inc. (Rockville, MD) and treated with dextran-coated charcoal at room temperature until <1% of [^{3}H]thymidine added before the procedure remained.

Propagation of cells in culture. ZR-75-B cells, a line of human breast cancer cells in continuous monolayer culture, were provided by Dr. Marc Lippman (National Cancer Institute). This is a cloned line from the previously described ZR-75-1 cell line (16) that has the same hormonal receptors and responsiveness as the parent line. The cells were grown in 75-cm^2 plastic flasks (Costar, Data Packaging, Cambridge, MA) in improved minimal essential medium (IMEM; National Institutes of Health Media Unit, Bethesda, MD) supplemented with 5% FCS, penicillin at 124 μg/ml and streptomycin 270 μg/ml under 5% CO_2 at 37°C. 3 d before MTX treatment, the cells were transferred to folate-free IMEM supplemented with 5% charcoal-treated FCS. Folate- and serum-free IMEM containing 2 mM L-glutamine supplemented with 10 μM thymidine (dT) and 10 μM deoxyinosine (dI) was used both during MTX incubation and during periods in drug-free medium following drug exposure to prevent cytotoxicity.

Characterization of DHFR-bound and -free intracellular MTX. To separate drug bound to DHFR from drug free in the cytosol, cells incubated with [^{3}H]MTX-Glu_1 were washed with ice-cold phosphate-buffered saline (PBS) and scraped off the flask surface with a rubber policeman in 1.5 ml of ice-cold KH_2PO_4 0.15 M, pH 6.2. Cell lysis was completed by sonication for 5 s on a Branson 350 sonicator (Branson Sonic Power Co., Danbury, CT) and 0.35 ml of the suspension was immediately added to 4.65 ml of 10% trichloroacetic acid (TCA) to precipitate macromolecules and dissociate bound drug from DHFR. After high-pressure liquid chromatographic (HPLC) separation of MTX-Glu_{1-5} (14), the total concentration of each compound was calculated in nanomoles per gram based on the radioactivity in the TCA supernatant, the specific activity of [^{3}H]MTX-Glu_1 used, and the amount of protein in the TCA precipitate as measured by Lowry protein assay (17). After pelleting cellular debris by centrifugation at 10,000 *g* for 5 min, the remaining cell lysate was immediately applied to a 0.8 × 2.5-cm DEAE-Sephacell minicolumn equilibrated with KH_2PO_4 0.15 M, pH 6.2, at 4°C. Protein-bound drug was first eluted at 4°C with 4 ml of the KH_2PO_4 buffer, following which unbound drug was recovered with 5 ml of 1 M NH_4HCO_3 (14). The total amount of drug present in each fraction was determined by multiplying the total drug measured in the TCA supernatant by the percent measured in each fraction. The eluate containing protein-bound drug was collected in TCA (final concentration 10%) to dissociate MTX and its metabolites from DHFR before HPLC quantitation of each compound in that fraction. The concentrations of MTX-Glu_{1-5} in the unbound fraction were determined by subtracting the amounts measured in the bound fractions from the total drug concentrations assayed in the TCA-treated samples. This method was used because breakdown of MTX-Glu_4 and Glu_5 to MTX was often observed on direct measurement of drug in the free fraction, ostensibly as the result of the effects of the enzyme folyl-polyglutamate carboxypeptidase in the cell lysate (9).

DHFR activity determination. DHFR activity was determined spectrophotometrically (18) in cell cytosol preparations after removal of MTX-Glu_{1-5} from the enzyme by dialysis. The cell extract was prepared by the following procedure: At the end of incubation with [^{3}H]MTX-Glu_1, the cells were lysed by scraping in 1 ml of 0.05 M Tris–HCl, pH 8.5, and cellular debris was pelleted by centrifugation at 10,000 *g* for 30 min. The cytosol preparation was then dialyzed at 4°C against 0.05 M Tris–HCl, pH 8.5, with 4.5 × 10^{-5} M FH_2 and 4 mM 2-mercaptoethanol across a membrane that allowed passage of molecules < 12,000 in molecular weight (Spectrapor 3787-D10; Arthur H. Thomas Co., Philadelphia, PA), and was continued until <5% of the predialysis counts of [^{3}H]MTX remained in the sample. Units of enzyme activity represent micromoles of NADPH consumed per minute. Both control and MTX-treated enzyme preparations were dialyzed under the same conditions. Control DHFR activities were similar to those previously measured in nondialyzed preparations (14).

RESULTS

Appearance of intracellular MTX-Glu$_{1-5}$ during drug exposure

We first monitored the appearance of MTX-Glu$_{1-5}$ both bound to DHFR and free in the cytosol during a 24-h incubation of ZR-75-B cells with 2 μM [^{3}H]MTX-Glu$_1$. After the 1st h of incubation, the cells had accumulated enough drug (10.1 mmol/g) to saturate their intracellular binding capacity, which was 2.92 nmol/g. At this time, 90.1% of the drug free in the cytosol and 85.5% of drug bound to DHFR was still in the form of parent drug, MTX-Glu$_2$ accounting for the remainder. After 6 h of incubation, total intracellular drug increased to 21 nmol/g protein, the increase being accounted for by synthesis of MTX-Glu$_{2-5}$, which now comprised 55% of free and 73% of bound drug, indicating rapid replacement of the parent drug on the enzyme. After 24 h incubation, total intracellular drug increased to 50.8 nmol, MTX-Glu$_{2-5}$ accounting for 85% of both the bound and free fractions.

The changes in the individual MTX polyglutamate species in the free and the bound fractions are illustrated in Figs. 1 and 2, respectively. Although the amount of free MTX-Glu$_1$ remained relatively constant over the 24-h period, the polyglutamates progressively increased, the Glu$_4$ and Glu$_5$ metabolites becoming the predominant free forms after 24 h, accounting for 34.5 and 27.5% of total free drug, respectively. These changes were mirrored in the bound fraction, as each newly synthesized metabolite rapidly replaced the parent drug and shorter chain metabolite on the enzyme. At the end of 24-h incubation, the Glu$_4$ and Glu$_5$ metabolites occupied 34 and 25% of the binding sites, respectively. In the period from 12 to 24 h of incubation with MTX-Glu$_1$, we consistently observed a two- to threefold increase in intracellular DHFR (see increase in binding capacity in Fig. 2). The amount of bound drug measured in the cells at the end of the 24-h incubation in this experiment (6.2 nmol/g) was 2.1 times the amount present at the end of the 1st h of incubation (2.92 nmol/g). This increase in binding capacity was confirmed by a similar twofold increase in DHFR activity in the cells at the end of the drug treatment (0.0036 U/mg) compared with controls (0.0018 U/mg). Consequently, in the last 12 h of drug incubation, the amounts of MTX-Glu$_{1-3}$ on the enzyme remained constant, while the amounts of MTX-Glu$_{4\&5}$ increased in concert with the increased DHFR binding capacity.

Disappearance of intracellular MTX and MTXPG after drug removal

At the end of the drug incubation period, MTX was removed from the extracellular fluid, and the cells were washed with PBS and placed in drug-free media (folate- and serum-free IMEM with 10 μM dT and dI) for an additional 24 h. We examined the rates at which MTX-Glu$_{1-5}$ decreased from the bound and free fractions during this efflux period. Intracellular levels of MTX-Glu$_{1-2}$ dropped rapidly. Only 16% of MTX-Glu$_1$ remained after the 1st h in drug-free medium, while MTX-Glu$_2$ levels dropped more slowly, 43% remaining after the 1st h but only 4% after 6 h. MTX-Glu$_3$ and Glu$_4$ levels decreased at an even slower rate, 17% of the former and 43% of the latter remaining after 24 h in drug-free medium, while MTX-Glu$_5$ continued to be synthesized as levels increased from 13.77 at the end of drug incubation to 21.57 nmol/g after 12 h, and remained at 18.77 nmol/g at 24 h, a net increase

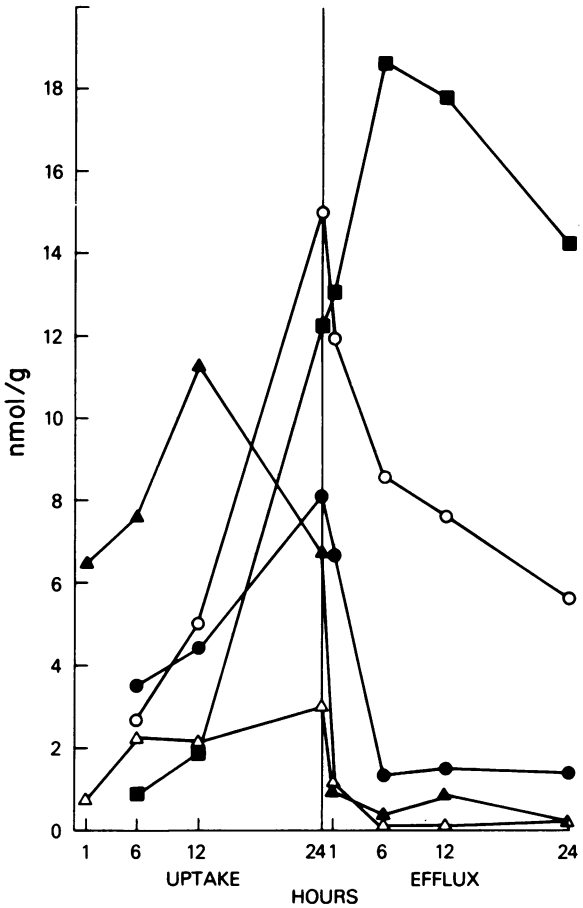

FIGURE 1 MTX and MTXPG free in the cytosol during drug exposure (uptake) followed by a period in drug-free medium (efflux). The concentrations of [^{3}H]MTX-Glu$_{1-5}$ in ZR-75-B cells free in the cytosol were determined during a 24-h incubation with 2 μM [^{3}H]MTX followed by a 24-h period in drug-free medium. Concentrations in nanomoles per gram are given at 1, 6, 12, and 24 h of drug uptake and after 1, 6, 12, and 24 h in drug-free medium. This experiment was repeated on two occasions and yielded the same pattern of results. MTX (▲); Glu$_2$ (△); Glu$_3$ (●); Glu$_4$ (○); Glu$_5$ (■).

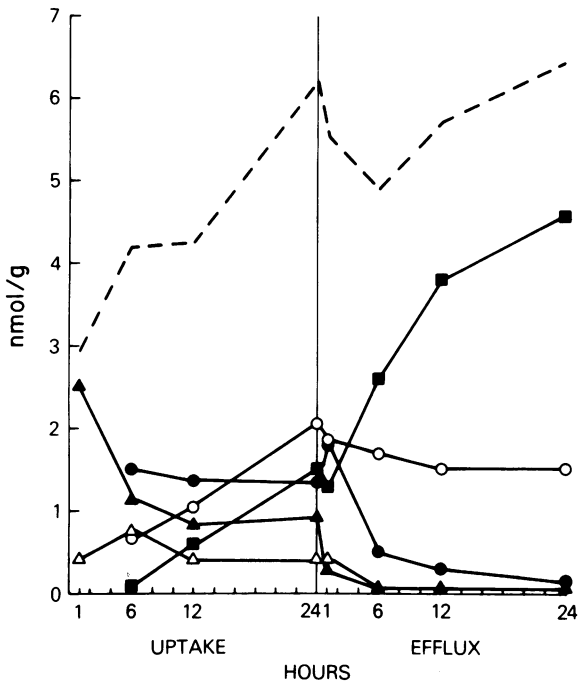

FIGURE 2 MTX and MTXPG bound to DHFR during drug exposure (uptake) followed by a period in drug-free medium (efflux). The concentrations of [^{3}H]MTX-Glu_{1-5} bound to DHFR in ZR-75-B cells were determined during a 24-h incubation with 2 μM [^{3}H]MTX followed by a 24-h period in drug-free medium. Concentrations in nanomoles per gram are given at 1, 6, 12, and 24 h of drug uptake and after 1, 6, 12, and 24 h in drug-free medium. Total drug-binding capacity (- - -); MTX (▲); Glu_2 (△); Glu_3 (●); Glu_4 (○); Glu_5 (■).

of 5 nmol/g. Since total intracellular drug decreased from 50.8 to 33.5 nmol/g during the period in drug-free medium, efflux from the cell was the predominant mechanism by which levels of MTX-Glu_{1-4} decreased.

The above changes in total intracellular drug were reflected by corresponding changes in both the bound and free fractions, as shown in Figs. 1 and 2. The rapid loss of MTX-Glu_1, -Glu_2, and -Glu_3 from the free fraction was accompanied by their disappearance from the bound fraction, and by a corresponding increase in the amount of bound MTX-Glu_5. Despite its 61% decrease in the free fraction after 24 h in drug-free medium, MTX-Glu_4 fell by only 23% (2.11–1.54 nmol/g) in the bound fraction. Free MTX-Glu_5 increased by 52% at 6 h and remained 16% above preefflux levels at 24 h. After 24 h in drug-free medium, the Glu_5 metabolite represented 71% of the bound drug.

Reversibility of the binding of MTX and MTXPG to DHFR

The foregoing studies suggested slower dissociation of MTX-Glu_4 from DHFR compared with MTX-Glu_{1-3} in drug-free medium. To determine the relative dissociability of MTX and MTX-Glu_{2-5} from DHFR, we examined the rates at which unlabeled MTX could replace [^{3}H]MTX-Glu_{1-5} on the enzyme. In the first phase of these experiments, ZR-75-B cells were first incubated in [^{3}H]MTX-Glu_1.

Experiment A. ZR-75-B cells were incubated in 2 μM [^{3}H]MTX-Glu_1 for 1 h followed by 1 h in drug-free medium (conditions under which the DHFR binding sites were occupied by [^{3}H]MTX-$Glu_{1\&2}$).

Experiment B. ZR-75-B cells were incubated in 2 μM [^{3}H]MTX-Glu_1 for 24 h followed by 24 h in drug-free medium (conditions under which the binding sites were occupied by MTX-Glu_{3-5}).

In the second phase of each experiment, the cells were resuspended in 10 μM unlabeled MTX-Glu_1 and the disappearance rates of [^{3}H]MTX-Glu_{1-5} from the protein-bound fraction were determined. The results are illustrated in Fig. 3. [^{3}H]MTX-$Glu_{1\&2}$ were readily replaced on enzyme binding sites by the unlabeled drug with half-lives of 12 and 30 min, respectively, while MTX-Glu_{3-5} were much less readily dissociable from the enzyme, with half-lives of 102, 108, and 120 min, respectively.

To verify that an excess of free unlabeled MTX-Glu_1 was present intracellularly during the second phase of each experiment, the concentrations of free labeled and unlabeled drug were measured at various time points during the competition phase of the experiment. The results are shown in Table I. 25% of the unlabeled drug was in the form of MTX-Glu_{2-3} after the 1st h of exposure (experiment A), while ~50% was in the form of MTX-Glu_{2-5} after 5 h (experiment B). In experiments A and B, during the competition phase of the study, the level of free unlabeled competitive MTX-Glu_1 exceeded the level of residual free labeled drug by ~30- to 85-fold in A and two- to threefold in B. When experiment B was repeated, but with a much higher concentration of unlabeled extracellular MTX-Glu_1 (400 μM), the ratio of intracellular MTX-Glu_1 to residual [^{3}H]MTX-Glu_{3-5} increased from 122- to 244-fold, but the off-rates of the various polyglutamate derivatives from DHFR were the same as when 10 μM unlabeled drug was used. Thus, the longer "off-rates" of MTX-PG were not an artifact of differences in the ratios of labeled to unlabeled drug, but reflected the difference in the rates of leaving the enzyme.

DISCUSSION

The present experiments have described the intracellular pharmacokinetics of MTX-Glu_1 and its polyglutamate metabolites, and have revealed marked differences in the formation, binding, and retention of the various polyglutamates in ZR-75-B cells. Previous studies have shown that the synthesis of polyglutamates up

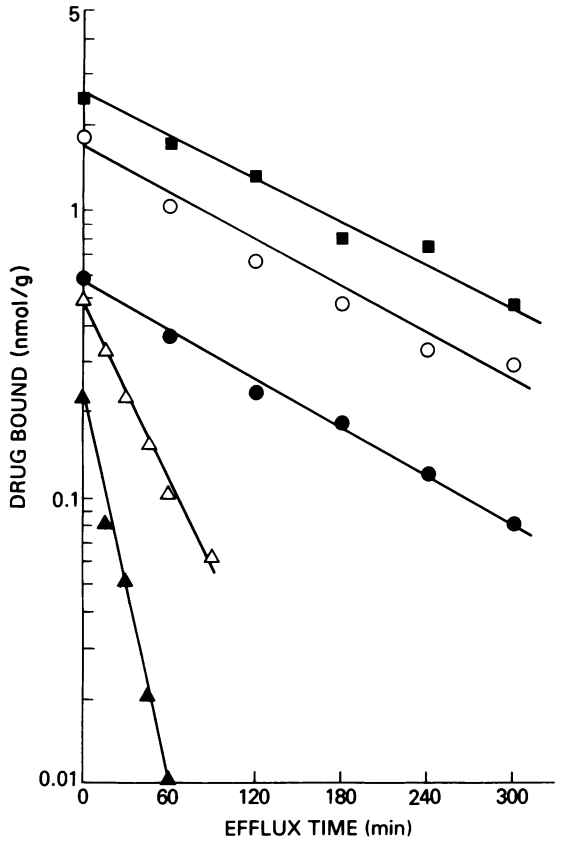

FIGURE 3 Dissociation curves of MTX and MTXPG from DHFR. The concentrations of [^{3}H]MTX-Glu$_{1-5}$ in ZR-75-B cells bound to DHFR were determined at the end of incubations with 2 μM [^{3}H]MTX-Glu$_1$ and following various periods (efflux) in medium containing unlabeled 10 μM MTX. The elimination half-lives for each compound ($t_{1/2}$) are: MTX-Glu$_1$ (▲), 12 min; MTX-Glu$_2$ (△), 30 min; MTX-Glu$_3$ (●), 102; MTX-Glu$_4$ (○), 108 min; MTX-Glu$_5$ (■), 120.

TABLE I
Dissociation of [^{3}H]MTX-Glu$_{1-5}$ from DHFR by Unlabeled MTX-Glu$_1$

Duration of unlabeled drug exposure	Unlabeled free drug	Labeled free drug	Unlabeled/labeled free drug
	nmol/g		
Experiment A			
15 min	42.8	1.5	28.5
30 min	88.3	1.8	49.1
45 min	100.6	1.2	83.8
60 min	93.4	1.1	84.9
Experiment B			
1 h	85.5	40.8	2.1
2 h	110.2	36.6	3.0
3 h	94.4	34.1	2.8
4 h	97.2	32.0	3.0
5 h	98.4	27.8	3.5

After a 1-h 2 μM [^{3}H]MTX incubation followed by 1 h in drug-free medium (experiment A) or a 24-h 2 μM [^{3}H]MTX incubation followed by 24 h in drug-free medium (experiment B), the cells were resuspended in 10 μM unlabeled MTX for 60 min in experiment A and 5 h in experiment B. The amounts of unlabeled and labeled free drug (nanomoles per gram) competing with the [^{3}H]MTX-Glu$_{1-5}$ on DHFR and the ratio of unlabeled to labeled free drug are shown at various intervals after the start of exposure to unlabeled drug. The amounts of unlabeled drug were determined in simultaneous experiments of identical design during which the initial 2-μM MTX exposures were done with unlabeled drug and the subsequent 10-μM MTX exposures with labeled drug.

to five glutamyl groups in length is dependent on both drug concentration and the period of incubation, the longer derivatives appearing only after several hours of drug exposure at concentrations of MTX-Glu$_1$ of 2 μM or greater (14). In this study, we have shown that the polyglutamate metabolites have a similar binding affinity for DHFR as they rapidly replace the parent compound in the enzyme-bound fraction, and do so in approximate proportion to their relative representation in the unbound cell cytosol.

These results are in agreement with those of Fry et al. (13) in Ehrlich ascites tumor cells, in which the same parameters were examined over a 4-h drug uptake period. Of greater importance, however, was the markedly prolonged retention of the longer polyglutamates—particularly MTX-Glu$_4$ and $_5$ in both the unbound and bound fractions after removal of extracellular drug—and their lower dissociability from the enzyme. Since studies with the parent drug have shown that it must be in excess intracellularly of the DHFR binding capacity in order to replace bound drug that dissociates from the enzyme (4), metabolism to MTX-Glu$_4$ or $_5$ would make the drug a much more efficient DHFR inhibitor.

On the basis of these findings, it is reasonable to conclude that the formation of MTX-Glu$_{2\&3}$ metabolites, which have similar intracellular pharmacokinetics to MTX-Glu$_1$, would not modify the drug's effect on DHFR. The Glu$_4$ and Glu$_5$ derivatives, however, are probably responsible for the prolonged inhibition of thymidylate synthesis previously observed in human breast cancer cells (14) and in other cell lines (8, 11). The published observation that MTX-Glu$_2$ is a more potent inhibitor of human thymidylate synthetase than MTX-Glu$_1$ (19) suggests that the formation and prolonged retention of MTX-Glu$_{4\&5}$ may extend the drug's effect to other folate-dependent enzyme systems, although it is not known which of the MTXPG metabolites has maximal inhibitory effect on this or other folate-dependent enzymes. These findings further suggest that polyglutamate formation, by extending intracellular half-life, prolonging the inhibition of DHFR, and changing the site of drug action could be an im-

portant determinant of cell sensitivity to MTX. Examination of this question will require studies in which tumor cell sensitivity to MTX is correlated with the ability of cells to form the various polyglutamate metabolites.

The prolonged drug exposure used in our experiments led to a twofold increase in the level of DHFR during the MTX incubation period. Although the amounts of bound MTX-$Glu_{4\&5}$ increased during the last 12 h of drug exposure, the amounts of bound MTX and lower MTXPG remained unchanged, possibly indicating that the rapidly increasing amounts of free MTX-$Glu_{4\&5}$ could bind newly synthesized enzyme. That the expanding drug-bound fraction represented DHFR was demonstrated by a corresponding increase of the enzyme activity measured by a spectrophotometric assay after drug removal from DHFR by alkaline dialysis. Increases in DHFR levels have been described previously during MTX therapy and were initially thought to result from enzyme stabilization by MTX (20), although more recent data suggest that MTX stimulates DHFR synthesis at the ribosomal level by an as yet undefined mechanism (21).

Our studies have shown that MTX-$Glu_{4\&5}$ have different properties than MTX-Glu_1. They are not only retained free in the cytosol much longer than the parent compound, but, as also described in rat hepatoma cells (22), the synthesis of MTX-Glu_5 continues in drug free medium at the expense of the shorter polyglutamates. They also appear to be significantly less dissociable from the target enzyme DHFR. These characteristics can explain the prolonged effects of MTX in human breast cancer cells even after the parent drug has been lost from both the intra- and extracellular milieu, and might be important determinants of the parent drug's antitumor activity.

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