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GROWTH INHIBITORY, TRANSPORT AND BIOCHEMICAL PROPERTIES OF THE γ-GLUTAMYL AND γ-ASPARTYL PEPTIDES OF METHOTREXATE IN L1210 LEUKEMIA CELLS *IN VITRO*

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Abstract—Both methotrexate-γ-glutamate and methotrexate-γ-aspartate are equivalent to methotrexate as inhibitors of L1210 cell dihydrofolate reductase. However, the initial influx of both peptides into L1210 cells during transport studies is substantially lower than that of methotrexate. The apparent K_m for influx of methotrexate-γ-glutamate and methotrexate-γ-aspartate is 15-fold and >100-fold greater than methotrexate respectively. Efflux measurements, which were possible only for methotrexate-γ-glutamate, showed a similar rate for this peptide and methotrexate. The intracelluiar accumulation and subsequent metabolism to methotrexate of methotrexate-γ-glutamate, but not of methotrexate-γ-aspartate, were confirmed by bioautographic analysis of cell extracts. After correction for extracellular cleavage of both peptides mediated by enzymes in calf serum supplementing the culture medium, the relative growth (L1210 cell)-inhibitory potency for the three agents was 1:18:210 for methotrexate, methotrexate-γ-glutamate and methotrexate-γ-aspartate respectively. Both the relative inhibitory potency and the difference in absolute inhibitory concentration among the three agents were predictable solely from the data on the influx of each measured during transport studies. Methotrexate-γ-aspartate is apparently more resistant to enzymic cleavage than is methotrexate-γ-glutamate.

It has recently been shown [1–3] that the folate analog, methotrexate, is metabolized at a significant level to the γ-diglutamyl form in a variety of mammalian tissues. Although higher polyglutamates of methotrexate are also found [1, 2] in these same tissues, the full extent of their formation has not been definitely established and, in contrast to the naturally occurring folates, may actually represent a smaller fraction. Because of the amount of methotrexate-γ-glutamate generated in tissue, an evaluation of its biological activity vis-à-vis methotrexate itself may be of potential therapeutic importance. Accordingly, this derivative was found by others to be less effective than methotrexate in inhibiting microbial growth [4], but equivalent to methotrexate as an inhibitor of dihydrofolate reductase from L1210 leukemia cells [3]. Moreover, it was found [3] to inhibit *in vitro* and *in vivo* the growth of these cells to a similar extent. As an extension of these studies, we now report on the transport of the methotrexate γ-glutamate derivative into L1210 cells. We also provide additional evidence relating to both dihydrofolate reductase and growth inhibition as well as to the metabolism of this derivative and the related γ-aspartyl peptide of methotrexate.

MATERIALS AND METHODS

Methotrexate was provided by Dr. Harry B. Wood, Jr., Division of Cancer Treatment, National Cancer Institute, Bethesda, MD. *N*-[*N*-[4-[[(2,4-diamino-6-pteridinyl) methyl]methylamino] benzoyl]-L-γ-glutamyl] -L-glutamic acid (methotrexate-γ-glutamate) and the analogous L-aspartic acid derivative (methotrexate-γ-aspartate) were synthesized by the following sequence. L-Glutamic acid α-benzyl ester was condensed with 4-[(benzyloxycarbonyl)methylamino]benzoyl chloride to give *N*-[4-[(benzyloxycarbonyl)methylamino]benzoyl]-L-glutamic acid α-benzyl ester, which was coupled at the unprotected γ-carboxyl grouping (mixed-anhydride method using *i*-butyl chloroformate) with dibenzyl L-glutamate and dibenzyl L-aspartate to give the blocked side-chain precursors. Catalytic hydrogenolysis gave the required *N*-[*N*-[4-(methylamino) benzoyl]-L-γ-glutamyl]-L-glutamic and L-aspartic acids. The tripeptide precursors thus prepared in unequivocal fashion were then alkylated by 6-(bromomethyl)-2,4-pteridinediamine [5] to give the desired products in high states of purity and free of methotrexate according to standard characterization methods and analysis by high-performance liquid chromatography. Each derivative was reexamined for purity by bioautographic analysis [6]. [^{3}H]methotrexate was purchased from Amersham/Searle and repurified by chromatography on DEAE-cellulose [7] or paper chromatography [6]. Dihydrofolate reductase was prepared [7] from ascites cell suspensions in BD2F$_1$ mice (A. R. Schmidt, Madison, Wisconsin) of a methotrexate-resistant L1210 leukemia provided by Dr. Dorris J. Hutchison,

Memorial Sloan-Kettering Cancer Centre, New York, NY. Inhibition of this enzyme was determined by a titration assay [8] measuring the effect of drug on the reduction of dihydrofolate [9].

Procedures for determining transport parameters of methotrexate and derivatives have been reported earlier [6, 10–12] and are outlined in the legend of Table 1. Intracellular drug accumulation was measured by titration assay [8] with a microbial dihydrofolate reductase [10]. In some experiments drug accumulation was measured [6] by radioactive counting of [^{3}H]methotrexate. The effect of methotrexate and derivatives on the growth of L1210 cells in culture was determined in RPMI 1640 medium (Grand Island Biologicals, Grand Island, New York) supplemented with 10% fetal calf serum (Microbiological Associates, Walkersville, Maryland). Details of the cell culture procedures have already been reported [13]. The cell line employed was derived from the same L1210 line V transplanted in BD2F$_1$ mice and used as a source of cells for transport studies. Further details as to the specific methodology employed in each experiment is given in the legend for each figure and table.

RESULTS

Prior work by others has already shown [3] that the γ-glutamyl derivative of methotrexate is as effective as methotrexate as an inhibitor of L1210 cell dihydrofolate reductase. We have been able to confirm this result and also find that the γ-aspartyl derivative exhibits an equivalent inhibitory potency. Our results are given in Fig. 1. In each case the inhibition observed is partially stoichiometric, and as

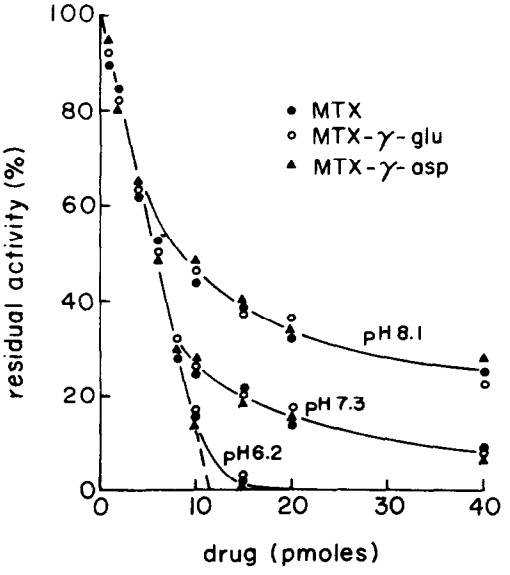

Fig. 1. Inhibition of L1210 cell dihydrofolate reductase by methotrexate (MTX), methotrexate-γ-glutamate (MTX-γ-glu) and methotrexate-γ-aspartate (MTX-γ-asp). Enzyme preparations were made from L1210 cells by sonication [6] of suspensions in 0.05 M Tris–HCl (pH 7.3) with 0.001 M EDTA. Varying amounts of drug were added to tubes containing 100 nmoles NADPH/ml, 1 mg/ml of mercaptoethanol in 0.05 M potassium phosphate buffer (pH 6.2, 7.3 or 8.1). Enough enzyme preparation was added to give a change in absorbance (A_{340}) of 0.3 in control tubes after incubation with 100 nmoles/ml of dihydrofolate for 10 min at 37°. The total volume of the reaction mixture was 2.5 ml. Methotrexate (100 μg) was added at the end of the incubation period to stop the reaction prior to the absorption measurement.

Table 1. Transport of methotrexate, methotrexate-γ-glutamate and methotrexate-γ-aspartate by L1210 leukemia cells*

	Influx kinetics				Efflux
Compound	V† (nmoles/min/mg dry wt) (N = 4)	V_{max}‡ (nmoles/min/mg dry wt) (N = 4)	K_m‡ (μM) (N = 4)	K_i‡ (μM) (N = 3)	k§ (N = 3)
Methotrexate	0.56	2.42 ± 0.7	3.3 ± 0.3	2.8 ± 0.6	0.204
Methotrexate-γ-glutamate	0.041	2.53 ± 0.5	49.3 ± 5.7	54.3 ± 8.4	0.228
Methotrexate-γ-aspartate	< 0.005		> 300.0		

* Leukemia cell ascites was removed from the peritoneal cavity of BD2F$_1$ mice, the cells were washed once in cold (0°) 0.14 M NaCl plus 0.01 M potassium phosphate (pH 7.4) and resuspended in transport medium [10] containing 107 mM NaCl, 5.3 mM KCl, 26.2 mM $NaHCO_3$, 1.9 mM $CaCl_2$, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM glucose and 10 mM Tris–HCl (pH 7.4). No serum was added. Initial influx measurements were made with varying external drug concentrations at 37°. The incubation time was adjusted at each drug concentration so that the intracellular level of drug accumulation never exceeded the dihydrofolate reductase-binding capacity. This allowed measurement of true unidirectional influx [10–12]. A double-reciprocal plot of the data (v/[drug]) was constructed to obtain values for maximum velocity (V_{max}) and the apparent Michaelis constant (K_m). Values for K_i were derived from similar data measuring the influx of [^{3}H]methotrexate obtained in the presence and absence of the competing analog and using the following calculation ($K_i = [I]/[K_p/K_m - 1]$ where K_p is the apparent K_m in the presence of inhibitor). Efflux of drug at 37° was measured [10] after resuspension of cells preloaded with drug in medium without drug. Initial efflux is essentially unidirectional because of the very large difference (> 300) in the size of the extracellular vs intracellular compartments. After influx or efflux measurements, incubation was terminated by 10-fold dilution of cells in cold (0°) buffered isotonic solution and washing three times with the same solution kept at 0°. No loss of drug occurs at this temperature [10, 12]. Also, since drug accumulated during influx is nonexchangeable (i.e. bound to dihydrofolate reductase), no loss would be expected during washing.

† $[MTX]_{ext} = 1.0$ μM.

‡ As determined during competition experiments measuring influx of [^{3}H]methotrexate.

§ First-order rate constant (min^{-1}).

shown earlier [3] the degree of stoichiometric inhibition is pH dependent. All three analogs were better inhibitors at pH 6.2 than at pH 8.1. It should also be noted that the same similarity in inhibition among the three analogs was observed for the microbial dihydrofolate reductase [9] employed in the titration assay for intracellular drug content.

Other results of a kinetic analysis of transport of methotrexate and both γ-glutamyl and γ-aspartyl derivatives of methotrexate are summarized in Table 1. A preliminary comparison of the initial influx velocity (v) for the three analogs showed that the rate of influx for the two peptides was considerably less than that observed for methotrexate itself. The γ-glutamyl derivative accumulated at a rate only $^1/_{14}$ of that seen for methotrexate and no rate determination could actually be made for the γ-aspartyl derivative. An analysis of the initial influx kinetics for methotrexate-γ-glutamate showed a similarity in the maximum velocity (V_{max}) for influx of this derivative and methotrexate. However, the value for the apparent K_m of the peptide was 15-fold (49.3 μM) greater than the value for methotrexate (3.3 μM). This large difference in the K_m value for the γ-glutamyl peptide compared to methotrexate was confirmed during competition experiments measuring [^{3}H]methotrexate uptake. The value for K_i derived (54.3 μM) was approximately the same as the K_m value. The amount of uptake of the γ-aspartyl derivative was so minute that a value for K_m could not be obtained. However, this was estimated to be somewhere above 300 μM, based on the highest concentration (100 μM) employed during determinations of initial influx velocity.

We considered the possibility that the kinetic constants for influx obtained for the γ-glutamyl derivative of methotrexate were, in fact, actually representative of the uptake of methotrexate generated by extracellular metabolism. Although the good agreement between the values for K_m and K_i would tend to exclude this possibility, a bioautographic analysis of drug in suspending medium after removal of cells was carried out. Only trace amounts of methotrexate (<1 per cent) could be identified in the supernatant fraction after incubation of cells with drug for as long as 10–15 min. This period of incubation is considerably longer than that normally employed (1–8 min) during the initial influx measurement at varying concentrations (see legend to Table 1). Moreover, the presence of such small amounts of methotrexate could not by itself account for the difference (15-fold) between the K_m values for methotrexate and methotrexate γ-glutamate actually observed.

Since the assay method employed does not distinguish between methotrexate and methotrexate-γ-glutamate, a more direct demonstration of methotrexate-γ-glutamate uptake was obtained by bioautographic analysis for intracellular drug content. However, in order to accumulate an adequate amount of drug, it was necessary to use a relatively prolonged incubation period at an external concentration of 100 μM. Under these conditions, intracellular levels of drug exceeded by a substantial amount the dihydrofolate reductase content. The results of such an analysis are shown in Fig. 2. After a 15-min incubation of cells with methotrexate-γ-glutamate, most of the intracellular drug actually exists as the peptide derivative. By 45 min a considerable amount of methotrexate is present along with the peptide, suggesting extensive metabolism within the intracellular compartment. In a similar analysis, the intracellular presence of methotrexate-γ-aspartate could not be demonstrated. When cells are preloaded in this manner for 15 min or less, it is also possible to estimate an efflux rate constant for methotrexate-γ-glutamate. The rate measured (see Table 1) was quite similar to that derived for methotrexate in a parallel experiment.

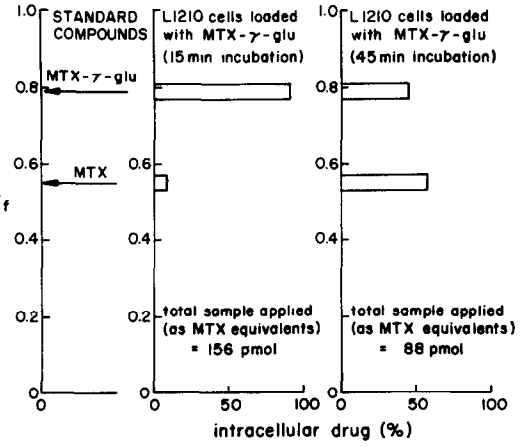

Fig. 2. Bioautographic analysis of intracellular drug after incubation of L1210 cells with methotrexate (MTX) and methotrexate-γ-glutamate (MTX-γ-glu). Cells suspended in transport medium [10] were incubated with 0.1 μmole/ml of drug for 15 or 45 min at 37°. The cells were washed three times in cold (0°) 0.14 M NaCl with 0.01 M potassium phosphate (pH 7.4) and resuspended in the same solution prior to the addition of an equal volume of cold 5% trichloroacetic acid (TCA) solution. Cell debris was removed by centrifugation and 0.1 ml extract spotted on 0.5-in. paper strips (Whatman No. 1). Chromatography was carried out in 1% sodium phosphate buffer (pH 8.1) for 5–6 hr at room temperature. Paper strips were removed, air-dried and placed on solidified agar medium [5] seeded with *Streptococcus faecium* var. *durans*. Agar plates were incubated 16–18 hr at 37° and the R_f of inhibition zones was determined. Zone size in mm was measured for the longest (L) and shortest (S) dimension, and the equivalence in drug determined from a standard curve prepared for each drug relating the product of L × S with amount. Methotrexate and methotrexate-γ-glutamate were prepared in TCA cell extracts and run as controls. Total recovery of drug (methotrexate and methotrexate-γ-glutamate) was 85–95 per cent of that amount determined to be present in extract by titration inhibition assay (Fig. 1).

The relative growth inhibitory potency of methotrexate and both peptide derivatives is shown in Fig. 3. Using an IC_{50} determination at 24, 48 and 72 hr, the potency against L1210 cells of the three agents varied over a >2-log range in the order, methotrexate ≥ methotrexate-γ-glutamate ≥ methotrexate-γ-aspartate. Although there was some decrease in the IC_{50} value for methotrexate within the time span employed, the decreases in the value for methotrexate-γ-aspartate and, to a larger extent, of methotrexate-γ-glutamate were greater. The decrease in the IC_{50} value for methotrexate can be

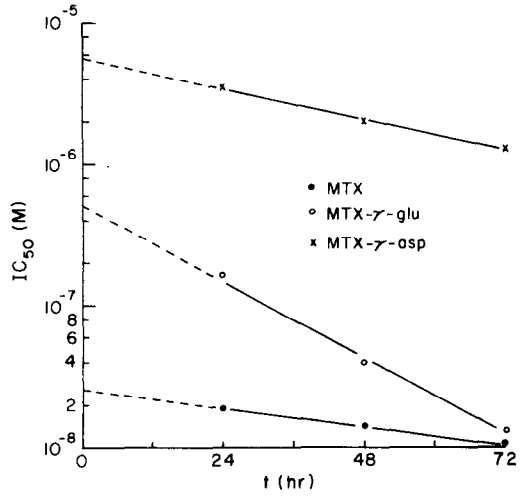

Fig. 3. Inhibition of L1210 leukemia cell growth by methotrexate (MTX), methotrexate-γ-glutamate (MTX-γ-glu) and methotrexate-γ-aspartate (MTX-γ-asp). Logarithmic phase cells (1.0×10^4 to 1.4×10^4) were inoculated into 5 ml (screw cap tubes) of RPMI 1640 medium supplemented with 10% fetal calf serum and varying concentrations of drug. After 24, 48 and 72 hr of incubation at 37°, cell number was determined by a Coulter Counter and the concentration of drug giving 50 per cent inhibition (IC_{50}) determined.

readily explained by a delay in the onset of inhibition which would distort the earliest determination to the greatest extent. Since the decrease observed with both peptide derivatives (the IC_{50} value for methotrexate-γ-glutamate at 72 hr actually approached that determined for methotrexate) was substantially greater, a study of the cleavage metabolism of the peptides was undertaken. The data shown in Fig. 4 were obtained by bioautography and represent the rate of appearance in the medium of methotrexate during growth of L1210 cell suspensions or in medium alone incubated for the same periods of time. The rate of appearance of methotrexate is

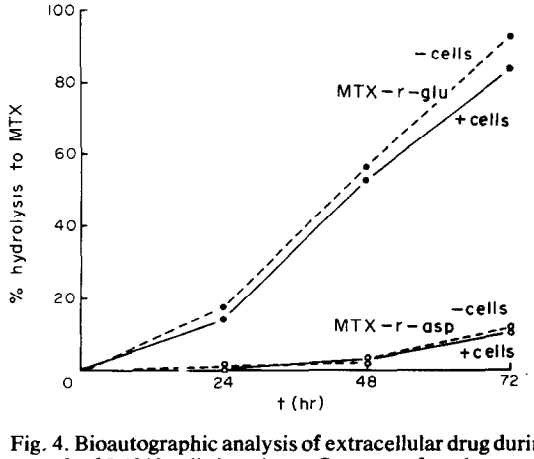

Fig. 4. Bioautographic analysis of extracellular drug during growth of L1210 cells in culture. Contents of methotrexate (MTX), methotrexate-γ-glu (MTX-γ-glu) and methotrexate-γ-aspartate (MTX-γ-asp) in cell culture supernatant solutions were compared to the content of the medium alone incubated for the same periods of time. Aliquots of 0.01 to 0.1 ml of medium were analyzed by procedures described in the legend to Fig. 2.

greatest in the case of methotrexate-γ-glutamate and, with both peptides, the rate of appearance of methotrexate was approximately the same in the presence or absence of growing cells. Moreover, a corresponding decrease occurred (data not shown) in the level of each peptide present. In other experiments it was also shown that the appearance of methotrexate in medium alone was associated with metabolism mediated by the serum fraction.

DISCUSSION

When the apparent extracellular metabolism of both methotrexate peptides is taken into consideration, the relative inhibitory potency between these peptides and methotrexate itself of L1210 cell growth can be explained solely by the observed kinetic differences in the transport of each. All three analogs are equally effective as inhibitors of dihydrofolate reductase. However, the influx of methotrexate-γ-glutamate (15-fold) or methotrexate-γ-aspartate (> 100-fold) is considerably less than that of methotrexate. Because of the log-linear relationship with time which was observed, back-extrapolation of the plots in Fig. 3 to zero time is possible and a relative estimate of the true inhibitory potency of the three analogs can be estimated. The ratios of potencies (1:18:210) obtained agree very well with the relative differences among individual values for K_m (influx) determined (methotrexate and methotrexate-γ-glutamate) or estimated (methotrexate-γ-aspartate) for each analog (Table 1). Moreover, by applying the basic Michaelis–Menten equation ($v = V_{max}$ [drug]/K_m + [drug]) for different drug concentrations and using a value of 12.5 ± 2 pmoles/min/g dry wt for the rate of dihydrofolate reductase synthesis at steady state logarithmic growth (enzyme binding equivalence = 9.0 ± 1.4 nmoles/g dry wt; cell doubling time = 12 hr), the actual external concentration of each analog necessary to achieve near maximum inhibition of growth can be calculated. Although these calculated values would be more appropriately related to measured values for IC_{90}, the values calculated for methotrexate and the -glutamyl and -aspartyl peptides (0.0165, 0.27 μM and > 2.0 μM respectively) are actually quite comparable to the true IC_{50} values estimated in Fig. 3.

From the data shown in Fig. 4, it appears that the γ-aspartyl derivative of methotrexate is a much poorer substrate for folate peptide cleavage enzymes than is the γ-glutamyl derivative. Moreover, in the face of evidence showing a decreased transport of methotrexate-γ-glutamate compared to methotrexate, the data in Fig. 4 appear to offer at least a partial explanation for the similar antileukemic equivalence and toxic potency of the same two derivatives observed [3] *in vivo* in mice. We have, in fact, been able to demonstrate (P. L. Chello and F. M. Sirotnak, unpublished results) peptide cleavage of methotrexate-γ-glutamate, but not of methotrexate-γ-aspartate, during incubation in undiluted mouse serum. It should also be noted that in an earlier [14] study of folate peptides, folate-γ-glutamyl-γ-aspartate was a poor substrate for liver γ-glutamyl carboxypeptidase.

Finally, it was of interest to note that the amino acid conjugation of methotrexate involving the γ-carboxyl group had little effect on the binding of this analog to dihydrofolate reductase, but had a profound effect on transport. In this connection, a very recent report [15] on the X-ray structure of the binary complex of methotrexate with purified dihydrofolate reductase provides related information. These workers suggest that the α-carboxyl group of the glutamate residue is hydrogen-bonded to arginine-57, but that the γ-carboxyl group in one of the two dihydrofolate reductase molecules in the asymmetric unit is hydrogen-bonded to an exterior solvent molecule, presumably H_2O. This conclusion is consistent with the binding of the γ-dipeptides to dihydrofolate reductase observed in this and earlier [3] studies. Information as to the ultimate therapeutic implications of these differences and the observation relating to the differential transport of γ-glutamyl and γ-aspartyl derivatives require further study. At the very least, these observations appear to have biochemical significance since they clearly suggest a difference in the structural basis for active site–drug interaction in each case.

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