STOICHIOMETRIC INHIBITION OF MAMMALIAN DIHYDROFOLATE REDUCTASE BY THE γ-GLUTAMYL METABOLITE OF METHOTREXATE, 4-AMINO-4-DEOXY-N^{10}-METHYLPTEROYLGLUTAMYL-γ-GLUTAMATE

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Summary - 4-Amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamate, a recently identified metabolite of methotrexate, was found to be equal in activity to methotrexate as an inhibitor of dihydrofolate reductase from murine L1210 leukemia cells, and as an inhibitor of the replication of L1210 cells in vitro and in vivo. The enzyme-inhibitory and cytotoxic activity of the metabolite was some 11-fold greater than anticipated from earlier studies utilizing microbiological assay systems. The results indicate that 4-amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamate formed in vivo may contribute significantly to the pharmacological activity of the parent drug.

The pteroylglutamate analog methotrexate* (4-amino-4-deoxy-N^{10}-methylpteroylglutamate) is a potent inhibitor of the enzyme dihydrofolate reductase (E.C. 1.5.1.3); the inhibition was characterized by Werkheiser (1) and by Bertino et al. (2) as "stoichiometric", i.e., one molecule of MTX is sufficient to inhibit one molecule of enzyme under appropriate assay conditions. Because of the tightness of binding of MTX within the enzyme:MTX:NADPH complex, rigorous kinetic studies have not been possible; present evidence indicates, however, that the inhibition is reversible and competitive with the substrate dihydrofolate, with a K_i of the order of 10^{-11} M (3).

Baugh and his co-workers (4) have recently shown that MTX is converted in vivo to its γ-diglutamate form, MTX(G_1); after administration of ^{14}C-labeled MTX to rats, some 25% of the drug in extracts from pooled viscera was detectable in the diglutamate form, while the greater part of the remaining

*The following abbreviations are used: MTX = methotrexate; MTX(G_1) = 4-amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamate; MTX(G_2) = 4-amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamyl-γ-glutamate; FH_2 = dihydrofolate.

radioactivity was attributable to unchanged MTX. Nair and Baugh (5) showed that MTX(G_1) is 11-fold less effective than MTX as an inhibitor of the growth of Streptococcus faecium; it was not determined, however, whether the lower effectiveness was attributable to lesser activity of MTX(G_1) as an inhibitor of S. faecium dihydrofolate reductase, or slower penetration of MTX(G_1) into bacteria. In contrast to naturally occurring compounds of the pteroylglutamate series, MTX did not undergo significant conversion to higher polyglutamates, although formation of a trace amount of MTX(G_2) could be demonstrated.

Because of the quantitatively significant conversion of MTX to MTX(G_1) in vivo, assessment of the activity of the latter compound as an inhibitor of mammalian dihydrofolate reductase has both theoretical and practical interest with respect to the biological activity of this widely used drug. The purpose of this study was to determine the activity of MTX(G_1) as an inhibitor of dihydrofolate reductase in both in vitro and in vivo assay systems.

MATERIALS AND METHODS

MTX(G_1) was synthesized by the solid-phase procedure of Nair and Baugh (5), and was purified by chromatography on DEAE-cellulose. 4-Amino-4-deoxy-N^{10}-methylpteroic acid used in the synthesis of MTX(G_1) was obtained by the enzymic hydrolysis of MTX, using carboxypeptidase G_1 from Pseudomonas stutzeri (6). Dihydrofolate reductase was purified 120-fold from L1210/A mouse leukemia cells (7,8); male BDF_1 mice bearing this tumor line were generously supplied by Dr. Dorris Hutchison, Sloan-Kettering Institute. Details of individual assay methods and experimental procedures are given in the legends for figures and tables.

RESULTS

Comparative effectiveness of MTX and MTX(G_1) as inhibitors of dihydrofolate reductase. The tightness of binding of dihydrofolate reductase by MTX was shown by Bertino and his co-workers (2) to be dependent on pH, with

"stoichiometric" inhibition being observed only toward the acid side of the physiological pH range. Because of this variation with pH, the enzyme inhibitory activity of MTX(G_1) was compared to that of MTX over a range of pH values; typical titration curves are shown in Fig. 1. MTX(G_1) was found to be

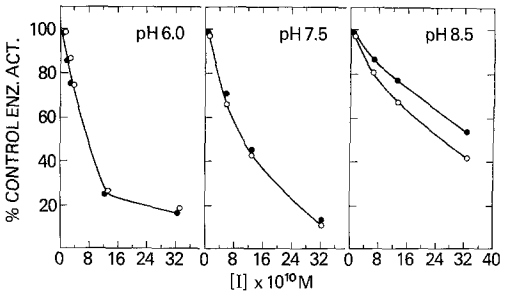

Fig. 1. Inhibition of mammalian dihydrofolate reductase by MTX (•) and by MTX(G_1) (o). Dihydrofolate reductase was purified 120-fold from mouse L1210/A leukemia cells by methods previously described (7,8). Cuvettes contained buffer, 100 µmoles (pH 6.0, sodium acetate; pH 7.5 and 8.5, Tris-HCl); potassium chloride, 150 µmoles; 2-mercaptoethanol, 15 µmoles; NADPH, 0.06 µmole; FH_2, 0.03 µmole; inhibitor (MTX or MTX(G_1)) at the concentration indicated on the abscissa; and sufficient dihydrofolate reductase to give a control (uninhibited) rate of 0.06 absorbance units/min at 340 nm, 25°, in a total volume of 1 ml. The reaction was initiated by the addition of FH_2, and reaction rates determined by means of a Gilford 2400 multiple sample absorbance recorder.

as effective as MTX as an inhibitor of dihydrofolate reductase at pH 6.0 and 7.5, and to be slightly more effective than MTX at pH 8.5. Like MTX therefore, MTX(G_1) is a stoichiometric inhibitor of the enzyme under appropriate assay conditions.

Inhibition of dihydrofolate reductase by MTX(G_1) formed from MTX in vivo. The primary evidence to date for the formation of MTX(G_1) in vivo consists of the observation that, in liver extracts from animals injected with ^{14}C- or ^{3}H-MTX, a peak of radioactivity has been found which co-chromatographs with authentic MTX(G_1) rather than with MTX (4,9). The biological activity of the metabolite has not, however, been established, and the presence of an endogenous inhibitor with chromatographic properties similar to those of pteroylpolyglutamates and MTX polyglutamates has recently been reported (10).

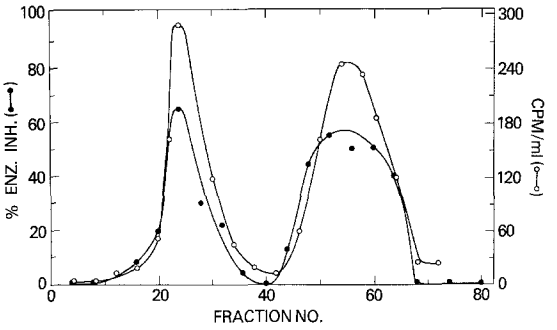

Fig. 2. Elution of ^{3}H-radioactivity (o) and of dihydrofolate reductase inhibitory activity (•) after application to Sephadex G-15 of liver extract from a male Sprague-Dawley rat pretreated with ^{3}H-MTX. A male Sprague-Dawley rat (250 g) was given 3',5'-^{3}H-MTX subcutaneously at a dose-level of 800 µg/kg daily for 3 days; total administered ^{3}H-radioactivity, 33.8 µCi. The animal was killed 24 hr after the third dose of ^{3}H-MTX, the liver removed, and an extract prepared by homogenizing the liver in 3 vol of 100 mM sodium phosphate buffer, pH 7.0, boiling for 5 min, centrifuging at 12,000 x g for 30 min, lyophilizing the supernatant fraction, and reconstituting in 3 ml sodium bicarbonate solution, 1%. A 1-ml aliquot was subjected to gel filtration on a column of Sephadex G-15 (2 x 21 cm) with a void volume of 30 ml. Elution was carried out with sodium phosphate buffer, 25 mM, pH 7.0; 3-ml fractions were collected and assayed for dihydrofolate reductase-inhibitory activity as described in Fig. 1, and for ^{3}H-radioactivity. Dihydrofolate reductase inhibitory activity: % inhibition observed with a 0.65-ml aliquot per column fraction, in a total assay volume of 1 ml. Subsequent to the latter determinations, the identities of Peak I (fractions 16 to 36) and Peak II (fractions 43 to 67) were further confirmed by rechromatography with authentic MTX(G_1) and MTX respectively. The experiment was repeated 3 times, with the data shown being obtained from a single representative experiment.

To determine whether the putative MTX metabolite exhibits enzyme-inhibitory activity identical to that of authentic MTX(G_1), rats were given 3',5'-^{3}H-MTX subcutaneously, liver extracts subjected to gel filtration on precalibrated Sephadex G-15 columns, and the fractions assayed both for ^{3}H-radioactivity and for ability to inhibit dihydrofolate reductase; detailed experimental conditions are described in the legend for Fig. 2. As shown, significant enzyme-inhibitory activity was detectable in both the MTX and the MTX(G_1) regions. The ratio of ^{3}H-radioactivity to enzyme-inhibitory activity in the two peaks (MTX and MTX(G_1)) was identical (Table 1). Since MTX(G_1) is equally as active as MTX as an inhibitor of dihydrofolate reductase under the assay conditions used (Fig. 1), this result supports the interpretation of

Table 1. Dihydrofolate reductase-inhibitory activity and ^{3}H-radioactivity in fraction 26 of Peak I (MTX(G_1)) and fraction 54 of Peak II (MTX) from the experiment illustrated in Fig. 2.

	MTX-equivalents (picomoles/ml)	^{3}H-radioactivity (dpm/ml)	Specific activity (μCi/μmole)
MTX(G_1) (fraction 26)	6.1	453	33.3
MTX (fraction 54)	5.2	423	36.6

MTX-equivalents were determined by inhibition titration with dihydrofolate reductase at pH 7.5, as described in the legend for Fig. 1.

Table 2. Inhibition of replication of L1210 cells in culture by MTX and MTX(G_1)

Inhibitor concentration (M)	Per cent inhibition in cell count/ml at 24 hr*		Per cent inhibition in cell count/ml at 48 hr†	
	MTX	MTX(G_1)	MTX	MTX(G_1)
4.4×10^{-9}	44	42	86	85
4.4×10^{-10}	31	11	64	68
2.2×10^{-10}	18	7	54	54

L1210 cells were maintained in static culture in RPMI #1630 medium with 20% fetal calf serum. For assay, stock cultures were diluted to 0.9×10^5 cells/ml. MTX and MTX(G_1) stock solutions were prepared in 1% sodium bicarbonate solution and were sterilized by filtration (Millipore SXGS filters) before addition to the assay media to yield the final inhibitor concentration shown. Cell counts were determined by means of a Coulter counter.

*Cell count in uninhibited control at 24 hr: 2.3×10^5 cells/ml

†Cell count in uninhibited control at 48 hr: 3.7×10^5 cells/ml

Baugh et al. (4) that both the radioactivity and the inhibitory activity in the MTX metabolite peak is attributable to MTX(G_1). In control studies with liver extracts from animals which had not received MTX, no significant enzyme inhibitory activity was detectable in the MTX or MTX(G_1) regions.

Table 3. Effect of MTX and MTX(G_1) on survival time of CDF_1 mice inoculated with L1210 leukemia cells.

Compound	Daily dose (mg/kg)	Average survival (days ± SE)
Controls (1% $NaHCO_3$)	-	9.3 ± 0.1
MTX	0.75	15.8 ± 0.8
MTX	1.25	18.6 ± 2.0*
MTX	1.5	16.7 ± 1.1
MTX(G_1)	1.0†	14.2 ± 1.5
MTX(G_1)	1.5	17.2 ± 0.8
MTX(G_1)	2.25	15.4 ± 1.3
MTX(G_1)	3.0	11.9 ± 1.1

Male CDF_1 mice, 10 per group, were inoculated i.p. with 2 x 10^5 L1210 cells. Treatment was started 24 hr later. MTX or MTX(G_1) were administered i.p. once daily for 10 days, in sodium bicarbonate, 1%.

*One long-term survivor (> 40 days) omitted.

†Dose-levels for MTX(G_1) expressed in MTX equivalents.

Inhibition of L1210 cell replication by MTX(G1). In view of the potent activity of MTX(G_1) as an inhibitor of dihydrofolate reductase from L1210 leukemia cells, it was felt to be of interest to determine its activity as an inhibitor of L1210 cell replication. As an inhibitor of growth of L1210 cells in culture, MTX(G_1) showed activity equal to that of MTX (Table 2). In vivo, MTX(G_1) was found to have antitumor activity similar to that of MTX in prolonging the life-span of CDF_1 mice bearing the L1210 leukemia: both drugs produced an approximate doubling of the life-span of treated animals as compared to the untreated controls (Table 3). The optimal dose-level of MTX, however, appeared to be slightly lower than that of MTX(G_1) (1.25 mg/kg vs. 1.5 mg/kg).

DISCUSSION

As an inhibitor of dihydrofolate reductase in vitro, MTX(G_1) was equally as effective as MTX (Fig. 1). This unexpected result supports the interpretation that the lower activity in microbiological assay systems previously reported for MTX(G_1) (5) was due to less effective cellular penetration of

the latter drug, rather than to a lesser ability to inhibit the target enzyme. The greater effectiveness of MTX(G_1) than of MTX as an inhibitor of the mammalian enzyme in the alkaline pH-range is not unprecedented; certain halogenated folate antagonists (e.g., 3',5'-dichloromethotrexate (11,12)) also exhibit this property, which, however, is not necessarily accompanied by greater *in vivo* activity.

It is of considerable interest that the enzyme-inhibitory activity of the MTX metabolite generated *in vivo* is identical to that of synthetic MTX(G_1) (Table 1). An alternative explanation for a peak of enzyme-inhibitory activity preceding the elution of MTX would have been the accumulation in liver of a naturally-occurring folate antagonist (e.g., N^{10}-formylfolate (10)) as an indirect consequence of the administration of MTX, but not arising as a metabolite of the latter. The identical ratios of ^{3}H-radioactivity to enzyme-inhibitory activity of the ^{3}H-MTX and ^{3}H-MTX(G_1) peaks separated by gel filtration (Table 1) tends, however, to make this possibility extremely unlikely.

The contribution of MTX(G_1) to the *in vivo* activity of MTX is not yet established, but the present studies suggest that it is not a biologically inert storage form of the latter drug.

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