Biochemical Pharmacology, Vol. 26, pp. 2310–2313. Pergamon Press, 1977. Printed in Great Britain.

Accumulation of methotrexate diglutamate in human liver during methotrexate therapy

(*Received* 1 *June* 1976; *accepted* 4 *March* 1977)

The antitumor agent methotrexate (MTX; 4-amino-4-deoxy-N^{10}-methylpteroylglutamate) is converted in rat liver to its γ-glutamyl conjugate, 4-amino-4-deoxy-N^{10}-methylpteroylglutamyl-γ-glutamate [MTX(G_1)] [1–4]. We have recently shown that the latter compound is a "titrating" or "stoichiometric" inhibitor of murine L1210 leukemia cell dihydrofolate reductase, being slightly more potent at physiological pH than the parent drug, MTX, in this respect [3]. We now report the presence of this biologically active conjugate in human liver obtained from patients receiving MTX as a therapeutic agent. A preliminary account of some of these studies has appeared in abstract form [5].

MTX(G_1) and [3′,5′-^{3}H]MTX(G_1) (sp. act. 2.6 mCi/μmole), for use as standard reference compounds in the biological studies, were synthesized by the method of Nair and Baugh [6]. The reference compounds [3′,5′-^{3}H]folic acid (sp. act. 15 mCi/μmole) and [3′,5′-^{3}H]MTX (sp. act. 5 mCi/μmole) were purchased from Amersham/Searle. Liver samples, 15–30 g, were obtained within 2 hr post-mortem from nine patients with neoplastic disease, who had received courses of MTX therapy within 6 months prior to death. Patients ranged in age from 21 to 69 years; the total dose of MTX received, the interval between the last dose and death, the level of MTX equivalents [MTX and MTX(G_1)] present in the liver and the percentage of the total present as the MTX(G_1) metabolite are shown in Table 1. In four patients, total MTX-equivalents were determined in the supernatant fraction from heat-treated liver homogenates by dihydrofolate reductase inhibition titration prior to separation of MTX and MTX(G_1) on precalibrated Sephadex G-15 columns, as previously described [3].

The identities of the MTX and MTX(G_1) peaks in the liver supernatant fraction were established by the following criteria. First, co-chromatography with the authentic reference compounds [3′,5′-^{3}H]MTX and [3′,5′-^{3}H]MTX(G_1) on Sephadex G-15. Second, inactivation of the dihydrofolate reductase-inhibitory activity attributable to the MTX and MTX(G_1) peaks, by incubation of the liver supernatant samples with rabbit liver aldehyde oxidase prior to Sephadex G-15 chromatography (Fig. 1). This experiment establishes that the activity attributed to MTX(G_1) could not be due to an endogenous folate derivative accumulating as a consequence of MTX therapy, since previous studies [7–9] have shown that 2-amino-4-hydroxypteridines, unlike 2,4-diaminopteridines, are not substrates for aldehyde oxidase. Third, co-chromatography of MTX(G_1) activity with authentic [^{3}H]MTX(G_1) on DEAE-Sephadex A-25 (Fig. 2). [3′,5′-^{3}H]Folic acid, 0.27 μCi, was used as an additional marker compound. This experiment establishes that the activity attributable to MTX(G_1) could not be due to the endogenous folate antagonist N^{10}-formylfolate, since the latter compound elutes prior to folate on DEAE-Sephadex A-25 [10], while the MTX(G_1) activity eluted after folate.

To determine whether MTX(G_1) retains its biological activity in man, dihydrofolate reductase from human lymphocytic leukemia cells was subjected to partial (640-fold) purification [11], and the ability of synthetic MTX(G_1) to inhibit this enzyme compared with that of the parent compound, MTX. By inhibition titration (Table 2), MTX(G_1) was found to be slightly more active than MTX as an inhibitor of human dihydrofolate reductase at pH 7.5.

With respect to the closely related enzyme thymidylate synthetase, Kisliuk *et al.* [12] have shown the pteroylpolyglutamates to have inhibitory activity, with pteroylglutamyl-γ-glutamyl-γ-glutamate being some 200-fold more effective as an inhibitor than pteroylglutamate. Since MTX is also known to be an inhibitor of this enzyme [13] it

Table 1. MTX(G_1) content of livers obtained post-mortem from nine patients receiving MTX therapy for neoplastic disease

Patient	Sex	Cumulative dose of MTX (mg)	Days from last dose of MTX to death	Total MTX-equivalents* [MTX + MTX(G_1)] (ng/g liver)	Per cent of MTX equivalents present as MTX(G_1)
S. T.	M	70	3		69
J. R.	F	4000†	12	44	34
N. M.	M	260	17	28	57
S. A.	M	5860†	24	60	47
J. F.	M	725	33		61
J. G.	F	150	44	46	42
H. K.	M	280	90		52
K. L.	F	600	165		> 99
K. P.	F	710	180		> 99

* Determined for livers from patients J. R., N. M., S. A. and J. G. only.
† MTX was given by a single high-dose infusion.

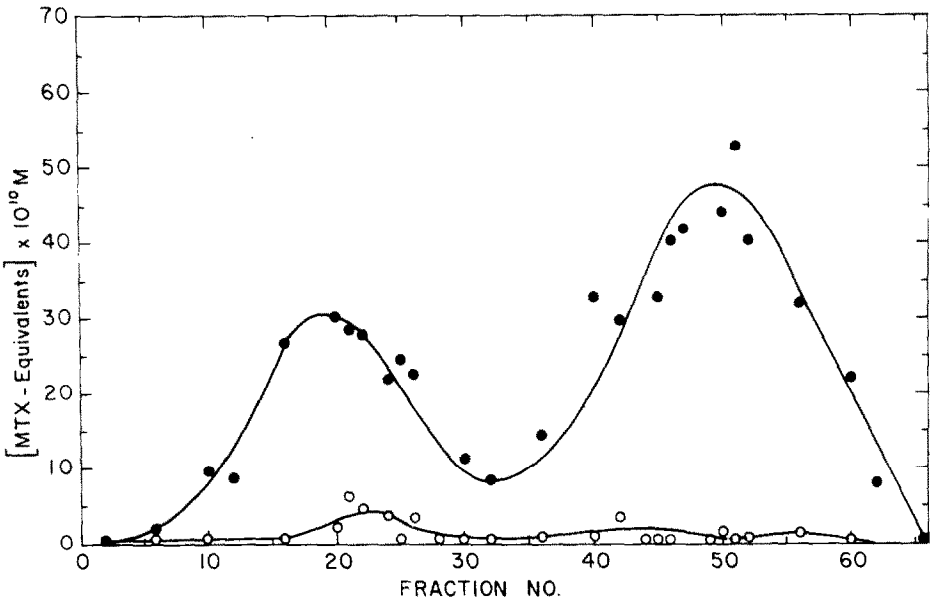

Fig. 1. Effect of rabbit hepatic aldehyde oxidase on Sephadex G-15 elution pattern of dihydrofolate reductase-inhibitory activity in liver sample from human subject treated with MTX. The liver supernatant fraction was prepared by homogenizing the liver sample in 3 vol. of 100 mM sodium phosphate buffer, pH 7.0, boiling for 5 min, centrifuging at 60,000 *g* for 30 min, lyophilizing the supernatant fraction and reconstituting in 6 ml sodium bicarbonate, 1%. A 0.8-ml aliquot was diluted to 1.0 ml with sodium phosphate buffer, 25 mM, pH 7.0, and subjected to gel filtration on a column of Sephadex G-15 (2 × 26 cm) with a void volume of 36 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 previously described [3]. An identical 0.8-ml aliquot of liver supernatant was added to sodium phosphate buffer, 100 μmoles, pH 7.0; Versene Fe-3, 15 μg; and partially (28-fold) purified rabbit liver aldehyde oxidase [8], 0.7 mg, in a final volume of 1.0 ml. After incubation for 3 hr at 37°, the sample was applied to an identical column of Sephadex G-15, fractions were collected as above and assayed for dihydrofolate reductase-inhibitory activity (○).

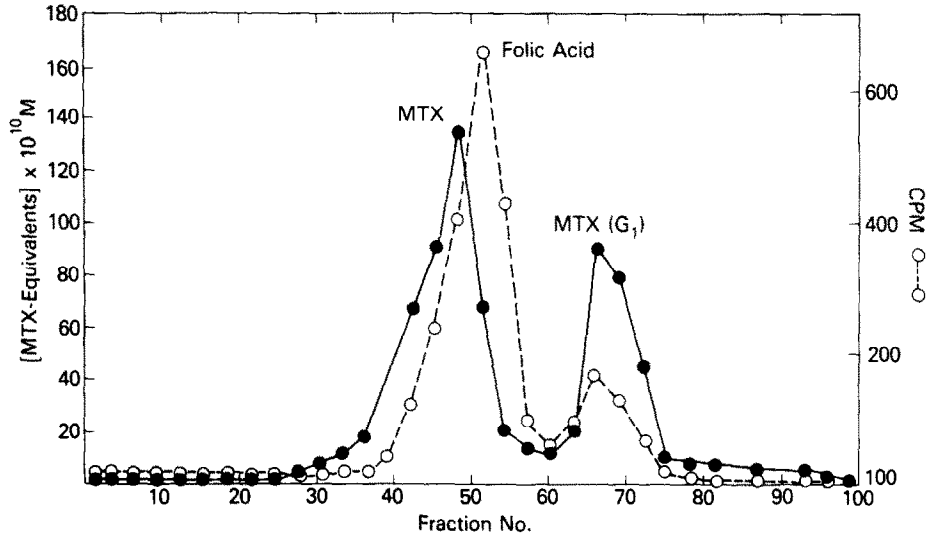

Fig. 2. DEAE-Sephadex co-chromatography of tritium-labeled reference compounds [3′,5′-^{3}H]folic acid and [3′,5′-^{3}H]MTX(G_1), with liver supernatant fraction from human subject treated with MTX. A 3-ml aliquot of liver supernatant prepared as described in Fig. 1 was mixed with 2 marker compounds, [^{3}H]folic acid, 0.27 μCi, and [^{3}H]MTX(G_1), 0.09 μCi, and diluted to a final volume of 10 ml which was applied to a column (1.5 × 16 cm) of DEAE-Sephadex A-25. Elution was carried out over a linear gradient from 0.1 M NaCl to 0.7 M NaCl in 0.01 M K_2HPO_4–KH_2PO_4, pH 7.0; 6.0-ml fractions were collected and assayed for dihydrofolate reductase inhibitory activity (●——●) [3] and for ^{3}H-radioactivity (○---○).

Table 2. Inhibition of dihydrofolate reductase from human acute lymphocytic leukemia cells by MTX and its γ-glutamyl conjugate, MTX(G_1)*

Inhibitor concn (nM)	Rate (Δ absorbance 340 nm/min)	
	I:MTX	I:MTX(G_1)
0	0.024	0.024
0.9	0.017	0.017
1.6	0.014	0.013
3.3	0.010	0.007

* Human dihydrofolate reductase was purified 640-fold from human acute leukemia cell lysate by affinity chromatography on a methotrexate-agarose gel column [8]. Assay cuvettes contained Tris–HCl buffer, pH 7.5, 100 μmoles; potassium chloride, 50 μmoles; 2-mercaptoethanol, 15 μmoles; NADPH, 0.06 μmole; inhibitor [MTX or MTX(G_1)] as indicated; and sufficient dihydrofolate reductase to give an uninhibited control rate of 0.024 absorbance units/min at 340 nm, 25°. Total volume of the reaction mixture was 1 ml. The reaction was initiated by the addition of dihydrofolate, 0.03 μmole, and reaction rates were determined by means of a Gilford 2400 multiple sample absorbance recorder. Each value is the mean of two determinations.

was felt to be of interest to determine the thymidylate synthetase inhibitory activity of its γ-glutamyl conjugate, MTX(G_1). MTX(G_1) was found to be several-fold more effective than MTX as an inhibitor of *Lactobacillus casei* thymidylate synthetase (Table 3). However, the pharmacological significance of the ability of MTX(G_1) to inhibit thymidylate synthetase is difficult to assess, since the experiments were not extended to human thymidylate synthetase, nor do the studies in post-mortem liver samples permit us to estimate the availability of intracellular MTX(G_1) [i.e. the ratio of unbound to dihydrofolate reductase-bound MTX(G_1)].

Since we were not able to obtain liver tissue from patients who received MTX at a lesser interval than 3 days prior to death, tissue levels of the conjugate during the period of maximal therapeutic activity of the drug are unknown; in the rat, however, MTX(G_1) is present in liver at a significant level (33% of total drug) within 4 hr after administration of MTX [4]. There appears to be a marked species difference in the persistence of the conjugate; the presence of MTX(G_1) for extremely long time periods in human liver (Table 1) is in contrast to the observation of Whitehead *et al.* [4] that, in the rat, MTX(G_1) disappears from kidney and liver relatively rapidly so that the conjugate is no longer detectable 26 days after administration of MTX. In man, MTX is detectable in human liver for several months after administration [15, 16] and since, as shown here, the γ-glutamyl conjugate of MTX has affinity of the same order of magnitude as the parent compound for human dihydrofolate reductase, the similar persistence of the conjugate in the form of MTX(G_1):dihydrofolate reductase:NADPH complex is not unexpected. The more rapid disappearance of MTX(G_1) in the rat may be attributable to higher liver peptidase activity or to faster rates of liver cell turnover in the latter species.

It is of interest that at the time periods studied here there appeared to be no relationship between total MTX dose and the amount of MTX retained in the conjugate form, i.e. an increase in MTX dose did not result in an increase in MTX(G_1) level (Table 1). Liver from the patients who received "high-dose" MTX therapy (J. R. and S. A.) did not show a greater percentage of the retained drug in the MTX(G_1) form than did liver samples from patients receiving conventional dose MTX therapy. In the rat [4], MTX polyglutamate formation appeared to be greater when the drug was given in frequent, divided doses, rather than in a single large dose. From this evidence, it would appear that the clinical regimen which would favor MTX polyglutamate formation would be continuous low-dose MTX treatment over long time periods. Although any association between MTX(G_1) accumulation and liver toxicity is purely speculative, it is noteworthy that studies on MTX-related liver disease in patients with psoriasis indicate that MTX when administered by a continuous low-dosage regimen has more serious sequelae than when given by an intermittent high-dosage regimen [17, 18].

Department of Medicine, SAMUEL A. JACOBS
Montefiore Hospital,
University of Pittsburgh School of Medicine,
Pittsburgh, PA 15213
Laboratory of Medicinal Chemistry and Biology, CHARLES J. DERR
DAVID G. JOHNS
National Cancer Institute,
Bethesda, MD 20014, *U.S.A.*

REFERENCES

1. C. M. Baugh, C. L. Krumdieck and M. G. Nair, *Biochem. biophys. Res. Commun.* **52**, 27 (1973).
2. Y. S. Shin, K. U. Buehring and E. L. R. Stokstad, *J. biol. Chem.* **249**, 5772 (1974).

Table 3. Inhibition of thymidylate synthetase from *L. casei* by MTX and its γ-glutamyl conjugate, MTX(G_1)*

5,10-Methylenetetrahydrofolate concn (μM)	Control [no MTX or MTX(G_1)]	MTX (10 μM)	MTX(G_1) (10 μM)
56	0.026	0.010	0.011
28	0.024	0.014	0.003
14	0.015	0.008	0.000
9	0.010	0.003	0.000

* Thymidylate synthetase, prepared from *Lactobacillus casei*, was provided by Dr. Charles Myers, National Cancer Institute. The standard assay mixture, described by Myers *et al.* [14], contained buffer, 37.5 μmoles Tris–HCl with 7.5 μmoles 2-mercaptoethanol, and 0.75 μmole EDTA, pH 7.4; undiluted cofactor solution composed of 0.22 μmole tetrahydrofolate, 15 μmoles formaldehyde, 25 μmoles $MgCl_2$, and 100 μmoles 2-mercaptoethanol, pH 7.4; inhibitor [MTX or MTX(G_1)] as indicated; and sufficient thymidylate synthetase to give an uninhibited control rate of 0.026 absorbance units/min at 340 nm, 37°, at a 5,10-methylenetetrahydrofolate concentration of 56 μM. The reaction was initiated by the addition of 0.05 μmole deoxyuridine monophosphate. The total volume of the reaction mixture was 1 ml, and the reaction rates were determined by means of a Gilford 2400 multiple sample absorbance recorder.

3. S. A. Jacobs, R. H. Adamson, B. A. Chabner, C. J. Derr and D. G. Johns, *Biochem. biophys. Res. Commun.* **63,** 692 (1975).
4. V. M. Whitehead, M. M. Perrault and S. Stelcner, *Cancer Res.* **35,** 2985 (1975).
5. S. A. Jacobs, C. J. Derr and D. G. Johns, *Proc. Am. Ass. Cancer Res.* **16,** 65 (1975).
6. M. G. Nair and C. M. Baugh, *Biochemistry* **12,** 3923 (1973).
7. D. G. Johns, A. T. Iannotti, A. C. Sartorelli, B. A. Booth and J. R. Bertino, *Life Sci.* **3,** 1383 (1964).
8. D. G. Johns and T. L. Loo, *J. pharm. Sci.* **56,** 356 (1967).
9. C. N. Hodnett, J. J. McCormack and J. A. Sabean, *J. pharm. Sci.* **65,** 1150 (1976).
10. M. d'Urso-Scott, J. Uhoch and J. R. Bertino, *Proc. natn. Acad. Sci., U.S.A.* **71,** 2736 (1974).
11. P. L. Chello, A. R. Cashmore, S. A. Jacobs and J. R. Bertino, *Biochim. biophys. Acta* **268,** 30 (1972).
12. R. L. Kisliuk, Y. Gaumont and C. M. Baugh, *J. biol. Chem.* **249,** 4100 (1974).
13. J. Borsa and G. F. Whitmore, *Molec. Pharmac.* **5,** 318 (1969).
14. C. E. Myers, R. C. Young, D. G. Johns and B. A. Chabner, *Cancer Res.* **34,** 2682 (1974).
15. J. R. Fountain, D. G. Hutchinson, G. B. Waring and J. H. Burchenal, *Proc. Soc. exp. Biol. Med.* **83,** 369 (1953).
16. S. Charache, P. T. Condit and S. R. Humphreys, *Cancer, N.Y.* **13,** 236 (1960).
17. M. G. C. Dahl, M. M. Gregory and P. J. Scheurer, *Br. med. J.* **1,** 654 (1972).
18. G. H. Millward-Sadler and T. J. Ryan, *Br. J. Derm.* **90,** 661 (1974).