In Vitro Formation of Polyglutamyl Derivatives of Methotrexate and 7-Hydroxymethotrexate in Human Lymphoblastic Leukemia Cells

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ABSTRACT

The intracellular synthesis of polyglutamyl derivatives of both methotrexate (4-amino-10-methylpteroylglutamic acid) and 7-hydroxymethotrexate, the primary plasma metabolite of methotrexate in humans, was evaluated in a methotrexate-sensitive, acute lymphoblastic leukemia cell line, MOLT 4. These studies were performed using a highly specific ion-pairing high-pressure liquid chromatography method which permits the simultaneous determination of methotrexate, 7-hydroxymethotrexate, and their corresponding polyglutamyl derivatives. When MOLT 4 cells were exposed to 1 μM methotrexate, the monoglutamate attained a steady state after 30 min, and polyglutamyl derivatives having from one to 4 additional glutamate residues were observed over 4 hr. Four additional metabolites were also detected upon incubation with 1 μM 7-hydroxymethotrexate. On the basis of the retention times for these compounds relative to net methotrexate polyglutamyl standards and since these metabolites reverted to 7-hydroxymethotrexate upon treatment with a preparation of hog kidney conjugase, they were identified as polyglutamyl derivatives of 7-hydroxymethotrexate. The identification of 7-hydroxymethotrexate polyglutamyl derivatives in vitro raises the possibility of an important new dimension in the pharmacological action of methotrexate. We investigated the effect of extracellular 7-hydroxymethotrexate on net methotrexate uptake and metabolism when cells were exposed simultaneously to 1 μM [3H]-methotrexate and unlabeled 7-hydroxymethotrexate. A decrease in the levels of both intracellular methotrexate and the corresponding polyglutamyl derivatives was noted for cells treated with 1 or 10 μM 7-hydroxymethotrexate. However, no appreciable effect of 7-hydroxymethotrexate on the amount of polyglutamyl derivatives formed relative to the total intracellular antifolate was noted. These studies show that in MOLT 4 cells (a) both methotrexate and 7-hydroxymethotrexate are rapidly converted to polyglutamyl derivatives, and (b) 7-hydroxymethotrexate interferes with net methotrexate accumulation and metabolism when present simultaneously in the extracellular medium. These results, moreover, suggest a potential role for 7-hydroxymethotrexate in modulating the biochemical effects of methotrexate in vivo.

INTRODUCTION

It was recognized in 1973 that MTX, an analogue of folic acid and a potent anticancer agent, was metabolized to polyglutamyl derivatives (3, 21). Subsequently, the conversion of the antifolate to polyglutamates was described in a wide variety of cells including rat hepatocytes (14), human fibroblasts (24, 25), human bone marrow (30), rat hepatoma (12, 13), the L1210 leukemia (29), the Ehrlich ascites tumor (9), human breast carcinoma (17, 27), and a human colorectal carcinoma cell line (5). The formation of these metabolites has been suggested to contribute to the prolonged pharmacological effects of MTX in vitro (17, 25, 26) and the selectivity of the antifolate in vivo (8).

Some factors that influence the accumulation and metabolism of MTX have been described. MTX accumulation and polyglutamylation are enhanced by the addition of glutamate or glutamine to the medium (2, 9). It has also been shown that the Vinca alkaloids (11) and probenecid (28) augment the steady-state level of free intracellular MTX in vivo. In addition, these compounds enhance the formation of MTX polyglutamyl derivatives (10).

In humans, MTX is significantly metabolized to form another derivative, 7-OH-MTX. Indeed, the plasma levels of this metabolite in patients have been reported to exceed those for MTX following i.v. infusion both at medium (18) and high doses (4, 7, 18) of MTX. Lankelma et al. (18) have shown that Ehrlich ascites tumor cells and human KB cells cultured in vitro with 7-OH-MTX exhibit decreased MTX transport. However, a detailed report of the effects of 7-OH-MTX on cellular accumulation and metabolism of MTX has not as yet been described.

In this study, we have investigated the net uptake and metabolism of both MTX and 7-OH-MTX in a MTX-sensitive, human acute lymphoblastic leukemia cell line, MOLT 4, using a highly specific HPLC analytical technique that allows not only the precise resolution of MTX and its polyglutamyl derivatives but also 7-OH-MTX and its corresponding polyglutamyl derivatives. In addition, we describe herein the effect of 7-OH-MTX on the net accumulation and polyglutamylation of MTX.

MATERIALS AND METHODS

Chemicals. [3',5',7-3H]MTX was purchased from Amersham-Searle Corp. (Arlington Heights, Ill.), and purified by a HPLC method using a C18-Bondapak column (Waters Associates, Milford, Mass.). The elution was carried out with a 25-min linear gradient from 1 to 25% acetonitrile in 5 mm sodium acetate buffer, pH 5.5. The flow rate was 2 ml/min. Purified synthetic MTX-G1, MTX-G2, and MTX-G3 were generously provided by Dr. J. Montgomery (Southern Research Institute, Birmingham, Ala.) and Dr. C. Baugh (University of South Alabama, Mobile, Ala.). Unlabeled 7-OH-MTX was prepared in our laboratory and purified according to the method of Jacobs et al. (15). Its purity was confirmed by UV absorption and by HPLC, with comparison to an authentic standard.

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† The abbreviations used are: MTX, methotrexate; MTX-Gn, methotrexate polyglutamates where n equals the number of additional glutamate residues, e.g., MTX-G1 is 4-amino-10-methylpteroylglutamyl-γ-glutamic acid.

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and kindly supplied by Dr. B. Chabner (National Cancer Institute, Bethesda, Md.). Other reagents were purchased from commercial sources and used without further purification.

**Enzymatic Preparation and Isolation of \( ^{[3H]}7\)-OH-MTX.** \( ^{[3H]}7\)-OH-MTX was prepared by the method of Johns and Loo (16). Fresh mature rabbit liver was homogenized in 2 volumes of double-distilled water. After centrifugation (15,000 \( \times \) g for 20 min), the crude supernatant fraction was removed and immersed in a water bath at 60° for 10 min. Heat-precipitated proteins were removed by centrifugation (1,000 \( \times \) g for 15 min), and the supernatant was mixed with an ammoniacal solution of ammonium sulfate prepared by adding 60 ml of an ammonium hydroxide solution (29%) to 940 ml of a saturated solution of ammonium sulfate. For each 100 ml of the supernatant, 59 ml of the solution of ammonium ammonium sulfate were added. After centrifugation (12,000 \( \times \) g for 15 min), the same amount of the ammonium sulfate solution was added to the supernatant, and the precipitate was recovered. The precipitate was then redissolved in 10% ammoniacal ammonium sulfate. Catalase (Sigma Chemical Co., St. Louis, Mo.) (1700 units), 6 ml of 0.4 m sodium phosphate buffer, and 17 ml of double-distilled water were added to the partially purified enzyme solution. \( ^{[3H]}7\)-OH-MTX was added dropwise while mixing at room temperature, and the solution was incubated overnight at 4°. Purification of \( ^{[3H]}7\)-OH-MTX was achieved by fracnating the preparation on a DEAE-cellulose column as described by Jacobs et al. (15).

The purity of the enzymically prepared radiolabeled 7-OH-MTX was confirmed by HPLC with comparison to an authentic standard of unlabeled 7-OH-MTX. An excellent correspondence between the unlabeled 7-OH-MTX marker (monitored at 254 nm) and the 7-OH-MTX radioactive derivatives was greater than 95%.

**Formation of Polyglutamyl Derivatives of MTX and 7-OH-MTX.** MOLT 4 cells were incubated 4 hr with 1 \( \mu \)M \( ^{[3H]}7\)-OH-FTX, and the total intracellular radioactivity present after 4 hr of incubation. Upon such a treatment, the late-labeled metabolites derived from MTX were polyglutamyl derivatives of MTX was obtained by an experiment in which samples from cells treated as described above were preincubated with hog kidney conjugase for 4 hr. Upon such a treatment, the late-labeled compounds reverted to a migration identical to that of MTX (Chart 1b).

**RESULTS**

**Polyglutamyl Derivatives of 7-OH-MTX.** Cells were incubated 4 hr with 1 \( \mu \)M \( ^{[3H]}7\)-OH-FTX, and the total intracellular radioactivity was quantitated and analyzed using the HPLC methodology described in "Materials and Methods." Five distinct radiolabeled metabolites could be demonstrated by this method (Chart 1A). The first peak (Peak 1) coeluted with authentic MTX and comprised nearly 24% of the total intracellular radioactivity present after 4 hr of incubation. Peaks 2 to 4 cochromatographed with MTX-G1 to MTX-G3, respectively. The retention time of Peak 5 is consistent with its identification as MTX-G4. Additional evidence that the radiolabeled metabolites derived from MTX were polyglutamyl derivatives of MTX was obtained by an experiment in which samples from cells treated as described above were preincubated with hog kidney conjugase for 4 hr. Upon such a treatment, the late-labeled components reverted to a migration identical to that of MTX (Chart 1b).

Similarly, cells incubated with 1 \( \mu \)M 7-OH-FTX formed 4 metabolites, the retention times for which on the chromatogram alternated with the MTX polyglutamate standards (Chart 2), suggesting that these components could be polyglutamyl derivatives of 7-OH-FTX. These derivatives were completely con...
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verted to 7-OH-MTX when exposed to the conjugase preparation as described above (Chart 1, C and D).

The time course of formation of MTX and its polyglutamyl derivatives is illustrated in Chart 3A. Within 30 min, MTX (Peak 1) had attained a steady state which persisted over the 4-hr interval of observation while a second small peak was observed which constituted approximately 12% of the total intracellular radioactivity and cochromatographed with authentic MTX-G1 (Peak 2). After 1 hr, 2 additional peaks accounting for 6.5 and 2.3% of the total intracellular radioactivity appeared and cochromatographed with MTX-G2 (Peak 3) and MTX-G3 (Peak 4), respectively. After a 4-hr incubation, MTX-G1 and MTX-G2 were the major intracellular forms of MTX. In addition, by 4 hr, another metabolite (Peak 5), presumably corresponding to MTX-G4, was detected, but its level remained extremely low, and the magnitude of its net accumulation was variable from experiment to experiment. After a 4-hr period of incubation with 1 mM MTX, 76.15 ± 4.6% (S.D.) (n = 3) of the total intracellular antifolate was present as polyglutamyl derivatives of MTX.

The time course for formation of the polyglutamates of 7-OH-MTX (1 µM, extracellular concentration) was also evaluated (Chart 3B). As for MTX, unmetabolized 7-OH-MTX (Peak 6) achieved a constant intracellular concentration by 30 min. There was a sequential appearance of the derivatives labeled 7 through 10 at 30 min, 1 hr, 90 min, and 150 min, respectively. After 4 hr, the predominant intracellular derivatives were those corresponding to Peaks 7 and 8, and the total extent of metabolism of 7-OH-MTX to polyglutamyl derivatives was 74.7 ± 13.28% (n = 3).

Effect of Extracellular 7-OH-MTX on MTX Accumulation and Polyglutamylation. The formation of MTX polyglutamyl derivatives was evaluated in the presence of different concentrations of 7-OH-MTX (0, 0.1, 1, and 10 µM) added in the extracellular compartment simultaneously with 1 µM [3H]MTX. In Chart 4 is reported the effect of 7-OH-MTX on both total MTX accumulation and the formation of polyglutamates after 4 hr of incubation. While the addition of 0.1 µM 7-OH-MTX had an insignificant effect on the net accumulation of total tritium (9.87 ± 3.0%; Chart 4B), higher concentrations of 7-OH-MTX reduced the level of intracellular antifolate achieved. The total intracellular MTX accumulation was decreased by 24.80 ± 4.58% (Chart 4C) and 46.26 ± 11.82% (Chart 4D) when cells were exposed to 1 and 10 µM 7-OH-MTX, respectively. This diminished intracellular drug concentration was accompanied by a corresponding reduction in the total level of the MTX polyglutamyl derivatives formed (36.56 ± 11.73% for 1 µM 7-OH-MTX and 53.51 ± 10.23% for 10 µM 7-OH-MTX).

DISCUSSION

The present report describes the formation of polyglutamyl derivatives of MTX and 7-OH-MTX in a MTX-sensitive, human acute lymphoblastic leukemia cell line, MOLT 4. Metabolism was evaluated by a new ion-pairing HPLC method which permits not only the resolution of MTX and its known metabolites, 7-OH-MTX and the MTX polyglutamyl derivatives, but also the previously unreported polyglutamyl derivatives of 7-OH-MTX.

MTX was found to rapidly form polyglutamates consisting of
1 to 4 additional glutamyl residues. After a 4 hr exposure to 1 \( \mu M \) MTX, 76.75 \( \pm \) 4.6% of the intracellular antifolate was present as the higher conjugates. A similar pattern was observed for the formation of the metabolites of 7-OH-MTX and on the basis of both the retention times for these compounds during HPLC relative to MTX polyglutamyl standards and their conversion to 7-OH-MTX upon treatment with a preparation of hog kidney conjugase, these metabolites have been identified as derivatives of 7-OH-MTX covalently associated with 1 to 4 glutamyl residues. Significantly, the rates and extent of formation of these metabolites of 7-OH-MTX were found to parallel those for the corresponding MTX conjugates (74.7 \( \pm \) 13.28% of total accumulation after 4 hr).

When 7-OH-MTX was evaluated for its effect on the formation of MTX polyglutamylderivatives, a significant reduction in the intracellular level of total antifolate was observed. Thus, as reported by Lankelma et al. (18) for the Ehrlich ascites tumor and human KB cells, 7-OH-MTX competes with MTX for binding to the membrane carrier for MTX transport. This reduction in the net accumulation of MTX was accompanied by the diminution in the intracellular levels of MTX polyglutamates. However, only a small reduction in the levels of polyglutamyl derivatives of MTX relative to the total intracellular drug was evident. Thus, while 7-OH-MTX interferes with MTX polyglutamylatuation, it appears that this is primarily a consequence of an effect at the level of the membrane carrier rather than the folypolyglutamate synthetase. Nonetheless, a competitive interaction between these compounds at the level of enzyme binding cannot entirely be excluded on the basis of the present data.

MTX polyglutamates have been demonstrated to form in both normal (14, 25, 26, 30) and neoplastic cell lines (5, 9, 12-14, 27, 28). These derivatives generally show an enhanced intracellular retention relative to the underivatized antifolate (1, 9, 13, 14, 17, 25, 26), thereby prolonging the biochemical effects of the drug (17, 25, 26) with increased cytotoxicity (13, 17, 25). The fact that the formation of MTX polyglutamyl derivatives may be an important element in drug selectivity is suggested by in vivo studies which indicate a low level of MTX polyglutamate formation in gastrointestinal epithelial cells but a significant accumulation of these metabolites in Ehrlich ascites tumors and L1210 leukemia cells (8, 22).

The inhibitory effect of 7-OH-MTX on accumulation of MTX derivatives demonstrated in these studies points to a potential role for the former compound in modulating the biochemical response to MTX. Indeed, following i.v. infusions with MTX in patients, 7-OH-MTX plasma concentrations have been reported to exceed those for MTX after 3 to 10 hr following infusion (4, 7, 18). When MTX plasma levels were in the range of 10^{-7} M, 7-OH-MTX levels were found to be 17 to 140 times higher than MTX (4). Thus, a competition between MTX and 7-OH-MTX at the level of the membrane transport carrier and/or at the folylpolyglutamate synthetase could influence the formation of these active polyglutamyl derivatives of MTX and, therefore, the cytotoxic effects of the antifolate.

However, preliminary animal studies indicate that 7-OH-MTX itself is not devoid of antifolate activity (23). While 7-OH-MTX has been shown to bind 2 orders of magnitude less effectively than MTX to dihydrofolate reductase (16), no assessment of the binding of the polyglutamyl derivatives of this compound has been reported. In addition, if 7-OH-MTX polyglutamates are formed as readily in vivo as demonstrated in this in vitro study, and if these derivatives are retained intracellularly as observed for MTX, these metabolites might accumulate to sufficient levels to inhibit dihydrofolate reductase even though they bind with a reduced affinity relative to MTX and its polyglutamyl derivatives.

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