Biochemical Pharmacology, Vol. 38, No. 5, pp. 815–822, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00
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FURTHER STUDIES ON THE PHARMACOLOGIC EFFECTS OF THE 7-HYDROXY CATABOLITE OF METHOTREXATE IN THE L1210 MURINE LEUKEMIA CELL*

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(*Received* 18 *April* 1988; *accepted* 25 *July* 1988)

Abstract—This paper describes studies that further explore the pharmacologic activity of the 7-hydroxy catabolite of methotrexate (7-OH-MTX). A 3-hr exposure of L1210 leukemia cells to 100 μM 7-OH-MTX produced negligible suppression of cell growth despite the build-up of intracellular polyglutamyl congeners to levels 2.7 times greater than the dihydrofolate reductase (DHFR) binding capacity. There was no evidence for direct inhibition of DHFR under these conditions based upon measurements of cellular tetrahydrofolate cofactor and dihydrofolate levels, nor was there suppression of [^{3}H]deoxyuridine incorporation into DNA or [^{14}C]formate incorporation into purines. When the interval of exposure to 100 μM 7-OH-MTX was increased to 6 hr, cell growth was inhibited by 60% and there was mild (~50%) inhibition of purine and thymidylate biosynthesis associated with a small increase in cellular dihydrofolate and a small decline in cellular tetrahydrofolates. Consistent with weak inhibition of DHFR was the absence of significant binding of 7-OH-MTX polyglutamates to DHFR as assessed by gel filtration of cell extracts. Mild direct inhibition of purine biosynthetics by 7-OH-MTX- or MTX-polyglutamyl congeners was demonstrated based upon inhibition of [^{14}C]formate incorporation into purines in cells pretreated with fluorodeoxyuridine so as to prevent tetrahydrofolate cofactor depletion or dihydrofolate polyglutamate build-up. Effects of a 6-hr exposure of cells to 100 μM 7-OH MTX on cell growth were reversed completely by 10 μM leucovorin; effects on cells containing comparable levels of MTX polyglutamyl congeners were unaffected by leucovorin. These studies demonstrate very weak inhibition of L1210 leukemia cell growth and purine, pyrimidine and tetrahydrofolate synthesis by the polyglutamyl congeners of 7-OH-MTX. The data suggest that effects of 7-OH-MTX polyglutamates on folate-requiring enzymes are not likely to play an important role in moderate-dose MTX regimens. However, pharmacologic activity may be expressed in high-dose MTX protocols when high blood levels of 7-OH-MTX are sustained over long intervals to the extent to which polyglutamate congeners accumulate in tumor cells and add to the much more potent inhibitory effects of MTX polyglutamates already present. Pharmacologic activity, however, would be diminished, if not completely reversed, by the concurrent administration of leucovorin.

A key factor in the cytotoxicity and selectivity of MTX§ [1–5] and the selectivity of leucovorin rescue [6–9] is the metabolism of MTX to polyglutamyl derivatives. MTX polyglutamates have high affinity for DHFR but, unlike the parent monoglutamate, they are retained within sensitive tumor cells to produce a sustained block in DHFR activity [2, 4, 5, 10, 11]. This results in the cellular depletion of tetrahydrofolate cofactors and the accumulation of dihydrofolate polyglutamates [8, 9, 12, 13] which, along with polyglutamates of MTX, inhibit target sites other than DHFR, including thymidylate synthase [14] and AICAR transformylase [15].

The presence of MTX polyglutamyl forms within cells is also a key element in preventing leucovorin rescue. Recent studies have shown that leucovorin rescue cannot be accounted for solely on the basis of provision of tetrahydrofolate cofactor substrate [8, 16–18]. Rather, leucovorin rescue appears to be based on multiple factors: (i) the net competitive displacement of monoglutamate antifolate from DHFR by leucovorin metabolites which results in reactivation of DHFR with repletion of tetrahydrofolate cofactor pools and reduction of cellular dihydrofolate polyglutamates [6–8], and (ii) the competitive interactions among tetrahydrofolate cofactors, dihydrofolate- and MTX-polyglutamates at the levels of thymidylate synthesis and purine transformylation [9, 14, 19]. In tumor cells that build up high concentrations of MTX polyglutamates, the extent and duration of inhibition of DHFR are increased, and net displacement of these derivatives from DHFR by leucovorin metabolites is negligible so that reactivation of DHFR is limited and dihydrofolate polyglutamate levels remain elevated [9, 18]. Thus, MTX polyglutamates sustain inhibition of DHFR and other sites, preventing utilization of the added tetrahydrofolate cofactor.

* This investigation was supported by USPHS Grant CA-39807 from the National Cancer Institute, National Institutes of Health.

† Supported by USPHS Training Grant CA-09340 from the National Cancer Institute, National Institutes of Health.

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§ Abbreviations: MTX, methotrexate; 7-OH-MTX, 7-hydroxymethotrexate; DHFR, dihydrofolate reductase; FdUrd, 5-fluorodeoxyuridine; AICAR, aminoimidazolecarboxamide ribonucleotide; and TCA, trichloroacetic acid.

MTX is also metabolized to the 7-OH-MTX catabolite in the liver of many mammalian species including humans [20, 21]. This derivative accumulates in the plasma after moderate to high doses of MTX, often exceeding that of the parent compound as the MTX level declines [22]. While the 7-OH-MTX catabolite has been considered to be an inert detoxification product of MTX [23], 7-OH-MTX has been shown to be transported rapidly into tumor cells by the MTX-folate cofactor carrier [24] and is metabolized rapidly to polyglutamyl derivatives by folylpolyglutamate synthetase [25, 26]. Accumulation of high cellular levels of the 7-OH-MTX polyglutamates results in cytotoxicity, but effects of these derivatives are considerably less potent than that of the MTX congeners [27, 28]. In this report we explore further the pharmacologic activity of 7-OH-MTX and, in particular, the effects of its 7-OH-MTX polyglutamyl derivatives on DHFR, thymidylate synthase and transformylation in purine biosynthesis within intact L1210 leukemia cells.

MATERIALS AND METHODS

Materials and chemicals. [3′,5′,7-^{3}H]MTX was acquired from Amersham (Arlington Heights, IL) and purified by liquid chromatography prior to use [11]. Unlabeled MTX, obtained from the National Cancer Institute, was purified by DEAE-cellulose chromatography [29]. [3′,5′-^{3}H]7-OH-MTX and unlabeled 7-OH-MTX were synthesized by direct hydroxylation of [^{3}H]MTX and unlabeled MTX, respectively, with a partially purified preparation of aldehyde oxidase (EC 1.2.3.1) obtained from mature rabbit liver [30]. The labeled and nonlabeled catabolite were purified by liquid chromatography [11] and DEAE-cellulose chromatography [29] respectively. Authentic MTX polyglutamate standards (4-NH_2-10-CH_3-PteGlu$_{2-4}$) were the gift of Dr. C. M. Baugh (Department of Biochemistry, University of South Alabama, Mobile). [3′,5′,7,9-^{3}H]Folic acid purchased from Amersham and [6-^{3}H]deoxyuridine purchased from New England Nuclear (Boston, MA) were purified by liquid chromatography [9].

Cells and culture conditions. The murine leukemia cell line, L1210, was maintained in continuous culture (shown to be mycoplasma free) in RPMI 1640 medium supplemented with 10% heat-inactivated undialyzed fetal calf serum, 2 mM L-glutamine, penicillin (100 units/ml), and streptomycin (100 μg/ml), all purchased from Gibco (Grand Island, NY), and 2-mercaptoethanol (20 μM). Suspension cultures in log growth had a constant doubling time of 12 hr.

For growth inhibition studies, cells (~10^6 cells/ml) were exposed to MTX or 7-OH-MTX for 1–6 hr, washed twice in unsupplemented medium, resuspended (~10^5 cells/ml) in complete medium containing dialyzed fetal calf serum, and incubated for 48 hr. Growth was monitored with a Coulter Counter (Coulter Electronics, FL) or by direct microscopic examination.

Analysis of drug uptake and metabolism. Studies on drug uptake and metabolism were performed in incubation flasks stirred with Teflon paddles and immersed in a 37° water bath. Washed cells were resuspended in complete RPMI 1640. A pH of 7.4 was maintained by passing warm humidified gas (95% air; 5% CO_2) over the cell suspension. Uptake was initiated by the addition of radiolabeled drug and terminated by injecting the cell suspension into 10 vol. of 0° 0.85% NaCl solution. The cells were collected by centrifugation and washed twice with the 0° saline solution.

One portion of the cell pellet was processed for total cellular drug. Cells were aspirated into the tip of a Pasteur pipet, extruded onto a polyethylene tare, dried overnight (70°), and weighed on a Cahn 4700 electrobalance (Cahn Instruments, Paramount, CA). The weighed pellet was digested in 0.2 ml KOH (1 N) for 60 min at 70° in a glass scintillation vial. After neutralization with 0.25 ml HCl (1 N), total radioactivity was measured in a liquid scintillation spectrometer.

The other portion of the cell pellet was analyzed for polyglutamate metabolites of MTX and 7-OH-MTX. Immediately following the cold wash, the remaining cell pellet was extracted with 1 ml TCA (10%) for 10 min at 0°. The acid extract was neutralized and chromatographed.

Liquid chromatographic analysis was performed on an Altex model 322 Liquid Gradient System (Beckman Instruments), equipped with an ODS analytical column (4.6 mm × 25 cm; IBM Instruments). The column was equilibrated with 0.1 M sodium acetate (pH 5.5); flow rate was 2.0 ml/min (2000 psi). The sample was eluted with acetonitrile (15%) using the following gradient: 0–38% over 0 to 2.5 min; 38–65% over 2.5 to 15 min; 65–100% over 15 to 15.2 min; 100% over 15.2 to 20 min. Fractions (1 ml) were collected and mixed with 3 ml Ready-Solv EP scintillation fluid (Beckman Instruments), and radioactivity was measured. Authentic standards of MTX-Glu$_{(1-4)}$ and 7-OH-MTX were included in the samples and monitored by UV detection (254 nm).

Analysis of cellular folate pools. Folate pools in L1210 cells, grown in folate-free medium with dialyzed fetal calf serum, were labeled with [^{3}H]folic acid (2 μM, 1000 dpm/pmol) for 2 days (four generations) prior to exposure to MTX or 7-OH-MTX. Cell doubling time remained at 12 hr under these conditions. Cell pellets washed with the 0° 0.85% saline solution were analyzed for total intracellular folates by a modification of the method of Matherly *et al.* [9]. Briefly, the washed cell pellet was suspended in nitrogen-saturated maleate buffer (0.1 M, pH 6) with mercaptoethanol (1%) and heated for 90 sec in boiling water in foil-covered tubes with serum stoppers evacuated with nitrogen. After rapid cooling to 0°, the extract was incubated for 60 min (37°) with hog kidney conjugase to cleave all folylpolyglutamates to their respective monoglutamates [31]. The recovery of 10-formyltetrahydrofolate (80%), 5-formyltetrahydrofolate (99%), 5,10-methenyltetrahydrofolate (18%) and 5,10-methylenetetrahydrofolate (~5%) under these conditions was assessed with radiolabeled standards. The recovery of 5-methyltetrahydrofolate and dihydrofolate was greater than 90%.

Deoxyuridine incorporation into DNA. L1210 leukemia cells were exposed to 10 μM MTX or 100 μM

7-OH-MTX for up to 6 hr in complete RPMI 1640 medium. Cells were washed twice and then were exposed to [6-^{3}H]deoxyuridine (3 μM; 500 dpm/pmol) in drug-free medium. At the intervals indicated, samples were removed, washed twice with 0° 0.85% saline, and then dispersed in 2 ml of 5% TCA (0°). The TCA precipitate was collected by centrifugation and then washed twice in 5% TCA (0°). Pellets were then transferred to polyethylene tares and processed as described above.

Formate incorporation into purine nucleotides and thymidylate. Following exposure of L1210 cells to MTX or 7-OH-MTX for 3 or 6 hr as described above, the cells were washed and resuspended in drug-free medium. The cells were incubated with [^{14}C]formate (100 μM; 20 dpm/pmol) and sampled at 10-min intervals.

Cells washed in the 0° saline solution were treated with perchloric acid (0.5 N) for 1 hr at 70° to hydrolyze purine and pyrimidine nucleosides and nucleotides to their respective bases. After cooling, the precipitated proteins were removed by centrifugation. The supernatant fractions were lyophilized, the residue was neutralized with KOH, and the potassium perchlorate precipitate was removed by centrifugation. The free bases quantitatively generated by this extraction were analyzed by liquid chromatography using an isocratic elution with sodium acetate (0.1 M, pH 5.5) for 8 min, followed by a linear gradient of 0 to 15% acetonitrile over 12 min [9]. Radioactive peaks were correlated with authentic base standards detected by absorbance at 254 nm. The observed retention times for the free bases guanine, thymine, and adenine were 7.0, 9.0, and 13.5 min respectively.

Analysis of drug binding to DHFR. Following incubation with radiolabeled drug, cells were harvested and washed as described above, and resuspended in 0° buffer (pH 6.0) containing 50 mM sodium citrate, 150 mM KCl, 50 mM mercaptoethanol, 1 mM EDTA, and 0.1 mM NADPH [11]. Following sonication (Fisher model 300) for two 20-sec periods (35% intensity with micro tip) in an ice bath, the sonicate was centrifuged (27,000 *g*, 4°) for 30 min to remove cellular debris; then 1-ml portions of sonicate were loaded onto minicolumns (5 ml) of Bio-Gel P6, (Bio-Rad Laboratories, NY). Preliminary studies established that bound drug was eluted by centrifugation (1000 *g*, 10 min), while free drug was retained completely on the column under these experimental conditions, as previously reported [32]. For analysis of the polyglutamyl profile of drug bound to DHFR, following gel filtration the eluents were extracted with TCA and chromatographed as described above.

RESULTS

Growth inhibition of L1210 cells by 7-OH-MTX polyglutamates; effect of leucovorin. L1210 leukemia cells were exposed to 100 μM 7-OH-MTX for 1–6 hr, washed, and resuspended into drug-free medium, and cell growth was monitored after 48 hr. Under these conditions, the parent monoglutamyl drug rapidly exits the intracellular compartment [25] so that only the polyglutamyl derivatives are retained to exert pharmacologic activity over the 2-day interval (4 doublings in untreated cells) of growth. Negligible inhibition of cell growth was observed for drug exposures up to 2–3 hr after which there was a progressive decrease in cell growth to 73, 62, 59, and 39% at 3, 4, 5 and 6 hr, respectively, as compared to control cells. For a 6-hr exposure to 7-OH-MTX the IC_{50} was 55 μM; the IC_{70} was 100 μM. In contrast, at 10 μM MTX, growth was suppressed completely; the IC_{50} was 1 μM after a 3-hr exposure to drug under these conditions.

Table 1. Leucovorin rescue from the pharmacologic effects of MTX or 7-OH-MTX

	Cell growth (% of control)	
	No leucovorin	With leucovorin
No drug	100	100
7-OH-MTX	31 ± 3	84 ± 3
MTX	0.7 ± 0.3	4 ± 1

Cells (~10^6 cells/ml) were treated with 7-OH-MTX (100 μM) or MTX (10 μM) for 6 hr, then washed and resuspended (~10^5 cells/ml) into drug-free medium with or without 10 μM leucovorin. Cell growth was monitored after 2 days. Untreated cells doubled four times and were assigned a value of 100%. The data represent the means of five to eight experiments ± SEM.

Leucovorin rescue was assessed in L1210 leukemia cells exposed to 10 μM MTX or 100 μM 7-OH-MTX for 6 hr and then grown in drug-free medium with or without leucovorin (10 μM) for an additional 2 days (Table 1). Leucovorin did not rescue cells containing high levels of MTX polyglutamates, whereas addition of leucovorin to cells containing 7-OH-MTX polyglutamates restored growth to levels nearly comparable to that of control cells.

7-OH-MTX polyglutamate formation as a function of duration of drug exposure. Exposure of tumor cells to 100 μM 7-OH-MTX results in the net accumulation of polyglutamyl drug levels which rapidly exceed the MTX binding capacity for DHFR. In agreement with previous reports [25, 28] the longest polyglutamyl derivative of 7-OH-MTX detected was the tetraglutamate (Table 2). After a 3-hr interval of exposure to 7-OH-MTX, the level of total polyglutamates was comparable to cells treated over the same interval with 10 μM MTX and exceeded the DHFR binding capacity by a factor of more than 2.7. Indeed, the level of the tetraglutamyl derivative alone was equal to the DHFR binding capacity. As the duration of incubation with monoglutamyl drug was increased to 6 hr, polyglutamates of 7-OH-MTX continued to accumulate such that the ratio of total polyglutamates to the DHFR binding capacity was increased to 7.4. Although there was an increase in the level of all the polyglutamyl forms, the greatest increase over this interval (~3-fold) was in the tetraglutamate derivative.

Effects of antifolate exposures on deoxyuridine incorporation into DNA. While the 7-OH-MTX polyglutamate level was several-fold higher than the DHFR binding capacity at 3 hr, there was a negligible effect on the incorporation of deoxyuridine into

Table 2. Comparison of polyglutamate accumulation after 3 or 6 hr of drug exposure

Drug metabolite*	7-OH-MTX 3 hr	7-OH-MTX 6 hr	MTX 3 hr
	(nmol/g dry weight)		
Glu_2	4.3	9.0	0.24
Glu_3	8.3	20.9	9.9
Glu_4	7.3	24.3	13.3
Total PGs	19.9	54.2	23.5
Ratio of total polyglutamates to DHFR binding capacity†	2.7	7.4	3.2

L1210 cells were exposed to 100 μM [^{3}H]7-OH-MTX or 10 μM [^{3}H]MTX for intervals of 3 or 6 hr, and polyglutamate formation was determined by liquid chromatography as described in Materials and Methods.

* Glu_n refers to the total number of glutamyl residues on the drug.

† The DHFR binding capacity for MTX in L1210 cells was 7.3 nmol/g dry wt. The data presented are from a representative experiment.

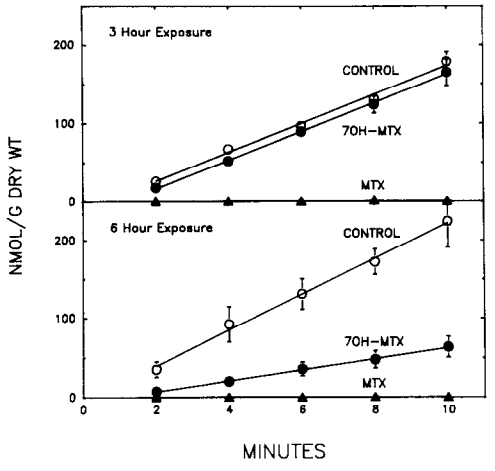

Fig. 1. Incorporation of [6-^{3}H]deoxyuridine into DNA in L1210 cells following a 3-hr (upper panel) or 6-hr (lower panel) exposure to antifolates. Cells were exposed to 7-OH-MTX (100 μM) or MTX (10 μM) for the specified interval, washed and resuspended into drug-free medium. Deoxyuridine (3 μM) incorporation into TCA insoluble cellular components was determined over a 10-min interval as described under Materials and Methods. Data represent the means of three experiments ± SEM.

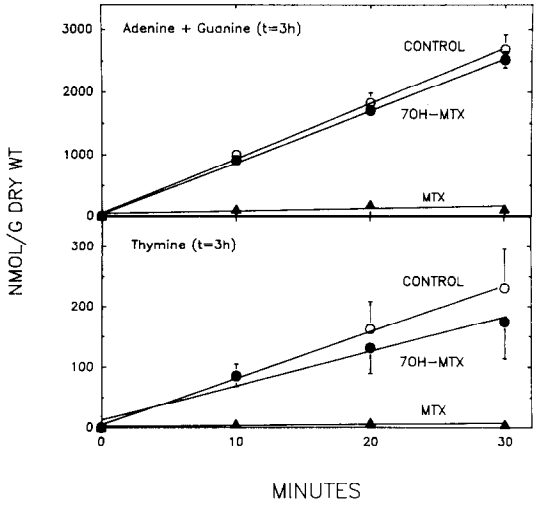

Fig. 2. Incorporation of [^{14}C]formate into total purines (adenine and guanine) and thymine in L1210 cells following a 3-hr exposure to antifolates. Cells were exposed to 7-OH-MTX (100 μM) or MTX (10 μM) for the specified interval, washed, and resuspended into drug-free medium. Cells were labeled with [^{14}C]formate (100 μM) for up to 30 min. Incorporation into purine nucleotides and thymidylate was quantitated by liquid chromatography following acid hydrolysis as described under Materials and Methods. Data represent the means of three experiments ± SEM.

DNA (Fig. 1, upper panel); incubation of cells for 3 hr with 10 μM MTX abolished deoxyuridine incorporation. However, as the duration of exposure to the antifolate was increased to 6 hr so as to accumulate even higher levels of 7-OH-MTX polyglutamates, deoxyuridine incorporation (Fig. 1, lower panel) into DNA was suppressed by 7-OH-MTX but much less so than by treatment with 10 μM MTX.

Effects of antifolate exposures on [^{14}C]formate incorporation into purines or thymidylate. [^{14}C]Formate combines with tetrahydrofolic acid to be incorporated into purines at the level of 10-formyltetrahydrofolate, or into thymidylate at the level of 5,10-methylenetetrahydrofolate. Following a 3-hr exposure to 10 μM MTX, formate incorporation into both purines and thymidylate was abolished (Fig. 2). In contrast, incubation of cells with 100 μM 7-OH-MTX for 3 hr produced no significant inhibition of these processes. However, as the level of 7-OH-MTX polyglutamates continued to accumulate in cells as the interval of exposure to drug was increased from 3 to 6 hr, one-carbon incorporation into purines and thymidylate was reduced to approximately 50% of control rates (Fig. 3).

One-carbon metabolism into purines was also assessed in cells pretreated with the thymidylate synthase inhibitor FdUrd. The concentration of FdUrd employed (3 μM) over 15 min completely prevented depletion of cellular tetrahydrofolates and

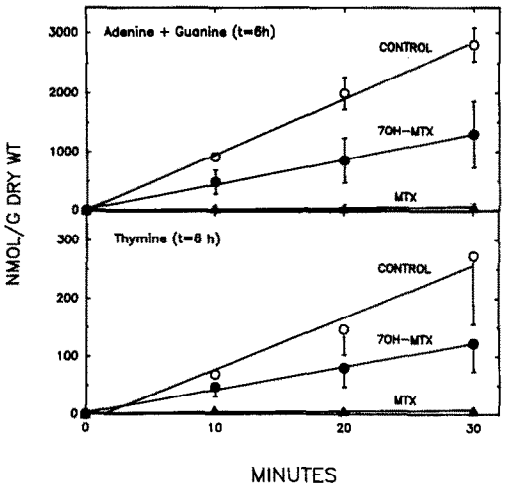

Fig. 3. Incorporation of [^{14}C]formate into total purines (adenine and guanine) and thymine in L1210 cells following a 6-hr exposure to antifolate, as described in the legend of Fig. 2. Data represent the means of three experiments ± SEM.

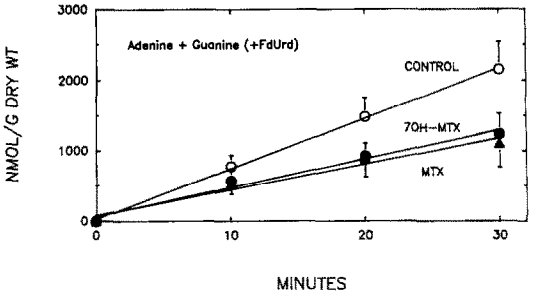

Fig. 4. Incorporation of [^{14}C]formate into total purines (adenine and guanine) in L1210 cells exposed to antifolate after treatment with FdUrd. Cells were incubated with FdUrd (3 μM) for 15 min prior to and during exposure to antifolate. [^{14}C]Formate incorporation into purines was measured as described in the legend of Fig. 2. Data represent the means of three experiments ± SEM.

the build-up of dihydrofolate polyglutamates (data not shown) during the subsequent exposure to 7-OH-MTX or MTX (Fig. 4). In the presence of FdUrd, the rate of incorporation of [^{14}C]formate into purines in control cells was unchanged. The suppression of [^{14}C]formate incorporation into purines by cells containing 7-OH-MTX polyglutamates was not altered by FdUrd. The inhibition of formate incorporation into purines by MTX, however, was diminished markedly by FdUrd pretreatment, becoming equivalent to that produced by 7-OH-MTX. FdUrd, under these conditions, inhibited [^{14}C]formate incorporation into thymidine by greater than 95% in control as well as antifolate-treated cells (data not shown).

Effects of 7-OH-MTX or MTX polyglutamates on endogenous folate pools. The effects of 7-OH-MTX- or MTX-polyglutamates on endogenous folate pools were assessed in cells equilibrated with [^{3}H]folic acid for 2 days prior to exposure to antifolates. When

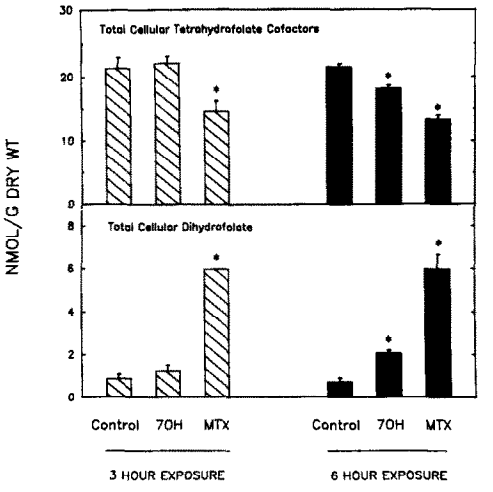

Fig. 5. Analysis of total cellular tetrahydrofolate (upper panel) and dihydrofolate (lower panel) cofactors in L1210 cells. Cells were labeled with [^{3}H]folic acid for 2 days followed by exposure to 100 μM 7-OH-MTX or 10 μM MTX for 3 or 6 hr. Folates were extracted and quantitated as described under Materials and Methods. Data represent the means of three experiments ± SEM. Asterisks (*) indicate statistically significant differences ($P \leq 0.05$; 4 df) from respective controls by Student's *t*-test.

the total tetrahydrofolate cofactor pool representing primarily 10-formyltetrahydrofolate, with lesser amounts of 5-formyl-, 5,10-methenyl-, and 5-methyltetrahydrofolate, was assessed in 7-OH-MTX-treated cells, there was no change compared to control cells at 3 hr, with only a 16% decrease at 6 hr (Fig. 5, upper panel). MTX treatment resulted in an approximate 35% decrease in total cellular tetrahydrofolates at both 3- and 6-hr exposures. The 10-formyltetrahydrofolate pools in control cells and in cells loaded with MTX or 7-OH-MTX polyglutamates for 6 hr were 15.7 ± 0.7, 7.5 ± 0.2 and 13.0 ± 0.9 (N = 3; mean ± SD) nmol/g dry wt respectively.

The effects of 7-OH-MTX polyglutamates on the dihydrofolate pool (Fig. 5, lower panel) were assessed as a sensitive indicator of drug effects on DHFR activity. After a 3-hr exposure to 7-OH-MTX, there was no significant increase in dihydrofolate above control levels. At 6 hr, the dihydrofolate level was elevated but to only one-third the level measured after treatment with MTX. MTX produced a partial interconversion of tetrahydrofolate pools to dihydrofolate, the extent of which was the same at 3 and 6 hr.

Analysis of antifolate polyglutamates bound to DHFR. The weak inhibition of DHFR produced in cells by 7-OH-MTX polyglutamates was consistent with low levels of binding of these congeners to DHFR, as assessed by gel filtration (Fig. 6). Cells were loaded with 10 μM MTX or 100 μM 7-OH-MTX for 6 hr to allow polyglutamyl derivatives to accumulate to levels far in excess of the DHFR binding capacity. The total amount of MTX monoglutamyl and polyglutamyl derivatives bound to DHFR represents the enzyme binding capacity [11].

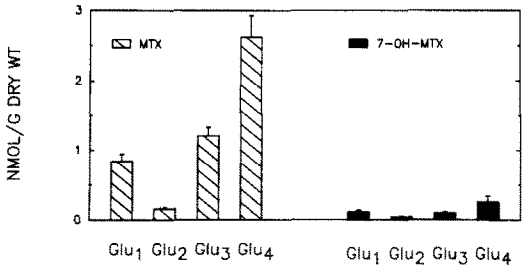

Fig. 6. Analysis of MTX polyglutamates or 7-OH-MTX polyglutamates bound to DHFR in L1210 cells. Cells were incubated with 10 μM [^{3}H]MTX or 100 μM [^{3}H]7-OH-MTX for 6 hr. The DHFR-bound drug was separated from the free drug component by gel filtration as described under Materials and Methods. Glu_n refers to the total number of glutamyl residues on the drug. Data represent the means of three experiments ± SEM.

In contrast, only very low levels of 7-OH-MTX polyglutamates could be detected bound to DHFR, representing only 17% of the MTX binding capacity, consistent with weak, reversible binding of 7-OH-MTX polyglutamates to this enzyme. Under these conditions, less than 15% of the DHFR binding capacity was associated with the monoglutamyl MTX; the rest was tightly bound by the di-, tri-, and tetraglutamyl MTX. The profile of MTX species bound to DHFR paralleled the levels of polyglutamates in the cytosol.

DISCUSSION

Previous studies from this and other laboratories have demonstrated that the 7-hydroxy catabolite of MTX interacts at many levels with biochemical processes that mediate the transport and metabolism of MTX and natural tetrahydrofolate cofactors. 7-OH-MTX has a high affinity for the reduced folate/MTX transport carrier [24], rapidly forms polyglutamyl derivatives [25] and, in the latter forms, can suppress folate-dependent enzymes such as DHFR [25] and AICAR transformylase in cell-free systems [29]. Since the drug accumulates to appreciable levels in the blood after moderate to high-dose MTX infusions [22], there is a potential for significant interactions with MTX, or direct effects of the 7-hydroxy catabolite, alone, that could influence the efficacy of high-dose MTX regimens that require leucovorin "rescue."

Data reported here indicate that, in comparison to MTX, the 7-hydroxy catabolite was a very weak inhibitor of L1210 leukemia cell growth under conditions in which high levels of intracellular 7-OH-MTX polyglutamates are present—far in excess of the DHFR binding capacity—consistent with a very low affinity for this enzyme. Second, whereas the cytotoxicity of MTX to L1210 leukemia cells that contain high levels of the polyglutamyl forms of this antifolate was affected minimally by leucovorin, the effects of 7-OH-MTX polyglutamates were reversed almost completely by this folate. Since leucovorin rescue has been shown to be due, to a large extent, to the reactivation of DHFR [8, 9], the ability of leucovorin to rescue cells that contain 7-OH-MTX polyglutamates is indicative of the low affinity of these derivatives for this enzyme and the ability of leucovorin metabolites to compete successfully with and reactivate DHFR even in the presence of high levels of these congeners of the catabolite.

Consistent with the weak inhibitory effects of 7-OH-MTX polyglutamates on cell growth were the minor effects on tetrahydrofolate-dependent reactions. Hence, after 3 hr of incubation with 100 μM 7-OH-MTX to achieve levels of polyglutamyl derivatives 2.7 times greater than the DHFR binding capacity, there was essentially no change in deoxyuridine incorporation into DNA or [^{14}C]formate incorporation into thymine, adenine or guanine. Consistent with this lack of effect on biosynthetic processes was a lack of effect on total cellular tetrahydrofolate cofactors and dihydrofolate, indicating no significant inhibition of DHFR within cells under these conditions. By 6 hr of exposure to 100 μM 7-OH-MTX with the build-up of polyglutamyl catabolites 7.4 times greater than the DHFR binding capacity, some suppression of DHFR was seen as manifested by a small increase in dihydrofolate substrate and a small decrease in total tetrahydrofolate cofactors. Accompanying this was weak inhibition of deoxyuridine incorporation into DNA and [^{14}C]formate incorporation in purines and thymidine. However, inhibitory effects were trivial in comparison to the marked inhibition of tetrahydrofolate-dependent purine and pyrimidine biosynthesis and the rise in cellular dihydrofolate and fall in cellular tetrahydrofolate cofactors obtained with one-tenth the level of MTX with the accumulation of comparable levels of intracellular polyglutamate derivatives.

While the pharmacologic effects of 7-OH-MTX on tetrahydrofolate-dependent biosynthetic processes were small and always accompanied by a fall in cellular tetrahydrofolate, the data suggest, in addition, a direct inhibition by 7-OH-MTX and MTX polyglutamates at the level of purine biosynthesis. Hence, when cells were pretreated with FdUrd, to prevent tetrahydrofolate cofactor depletion and dihydrofolate build-up, while the anti-purine effects of MTX were decreased markedly but not abolished, the anti-purine effects of 7-OH-MTX were sustained. This was consistent with a weak but separate direct inhibitory effect of 7-OH-MTX polyglutamates at the level of transformylation—consistent with the inhibition by 7-OH-MTX polyglutamates of AICAR transformylase in a cell free system [33]. The observation that inhibitory effects of MTX were reduced markedly by pretreatment with FdUrd, despite high levels of MTX polyglutamates in the cells, suggests that dihydrofolate polyglutamates that build up in its presence and the tetrahydrofolate depletion that occurs in cells in which thymidylate synthase is active results in the suppression of this pathway observed when thymidylate synthesis is unperturbed. It is clear from other studies that cellular MTX polyglutamates directly inhibit transformylation based upon effects on one-carbon flows into purines upon exposure of cells to radiolabeled leucovorin [9]. Hence, the residual suppressive effect of MTX on [^{14}C] incorporation into purines in cells pretreated with FdUrd

must reflect the direct though weak inhibitory effect of MTX polyglutamyl congeners on 10-formyltetrahydrofolate utilization in purine biosynthesis.

The data suggest that, in moderate-dose clinical regimens [34] with MTX (i.e. in the range of 100–200 mg/m^2), the 7-hydroxy catabolite has little direct pharmacologic importance. On the other hand, it is possible that in high-dose regimens in which very high plasma levels ($\geq$100 μM) of 7-hydroxy MTX are sustained for very long intervals (in excess of 24 hr [22, 35]), pharmacologic activity could be expressed to the extent to which these congeners (i) continue to accumulate in tumor cells with time, (ii) add to the much more potent inhibitory effects of MTX polyglutamyl derivatives retained in these cells and (iii) do not diminish or completely negate the concurrent administration of leucovorin.

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