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Inhibition of folate-dependent enzymes by 7-OH-methotrexate

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Methotrexate (MTX-Glu_1*; 4-NH_2-10-CH_3-pteroyl glutamate), a widely used antineoplastic agent, is thought to exert its antitumor effect by virtue of its tight-binding inhibition of dihydrofolate reductase (EC 1.5.1.3; DHFR) [1]. MTX has been shown to undergo intracellular polyglutamation in a manner similar to the polyglutamation of physiologic folates [2]. With a long intracellular $T_{\frac{1}{2}}$, these MTX polyglutamates (PGs) become the predominant form of intracellular drug in malignant cells and, to a lesser extent, in normal tissues. The MTX-PGs retain the inhibitory potency of the parent compound for dihydrofolate reductase [3]. Polyglutamated forms of MTX are also potent inhibitors of the folate-requiring *de novo* pyrimidine synthetic enzyme thymidylate synthase (EC 2.1.1.45; TS) [4] and aminoimidazole-carboxamide ribonucleotide transformylase (EC 2.1.2.3; AT) [5], one of two folate-requiring enzymes involved in *de novo* purine synthesis.

7-OH-MTX is the major metabolite of MTX, in which the 7-position of the pteridine ring is hydroxylated by aldehyde oxidase. This metabolite was first described by Johns and Loo in rabbits and was found to have intermediate potency as an inhibitor of DHFR, with a K_i of 6.6×10^{-9} M [6], or 2–3 orders of magnitude less than MTX. However, the metabolite is found in high concentration in plasma, particularly in patients treated with high-dose MTX, and becomes the predominant drug form 10–12 hr after MTX treatment [7, 8]. In a study of patients treated with high-dose MTX (140–350 mg/kg), the plasma concentration of 7-OH-MTX exceeded that of MTX 3–10 hr following the infusion of high-dose MTX [9]. At later time points, when plasma levels of MTX were in the range of 10^{-7} M, the level of 7-OH-MTX was found to be 17–140 times higher than that of MTX, and the plasma half-life of 7-OH-MTX was found to be 23.8 hr, or about three times longer than the terminal $T_{\frac{1}{2}}$ for MTX.

7-OH-MTX has been found to be polyglutamated intracellularly *in vitro* in the human acute lymphoblastic leukemia cell line (MOLT 4) at approximately the same rate and extent as MTX [10]. In Ehrlich ascites cells, the rate of polyglutamation of 7-OH-MTX exceeded that for MTX by a factor of 2.7 at equimolar extracellular drug levels, and 7-OH-MTX has been shown to be transported more efficiently than MTX in these cells [11].

Aside from their inhibition of DHFR, 7-OH-MTX and its polyglutamates have not been shown to be inhibitors of other folate-requiring enzymes. In this paper, we report the potencies of 7-OH-MTX and its tetraglutamated form as inhibitors of the *de novo* pyrimidine synthetic enzyme TS and the *de novo* purine synthetic enzyme AT.

Materials and methods

Chemicals. AICAR, 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide, 2-mercaptoethanol type I, dextran (clinical grade), deoxyuridine monophosphate (dUMP), bovine serum albumin fraction V, acid-washed activated charcoal, folic acid (crystalline), and L-ascorbic acid were purchased from the Sigma Chemical Co. (St. Louis, MO). Purified folic acid pentaglutamate ($PteGlu_5$) was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD). A Bio-Rad Protein Assay kit was purchased from Bio-Rad Laboratories (Richmond, CA). [5-^{3}H]dUMP (20 Ci/mmol) was obtained from the Moravek Biochemical Co. (Brea, CA). 7-OH-MTX-Glu_1 and 7-OH-MTX-Glu_4 were prepared and purified by high-pressure liquid chromatography according to published methods [12]. All other chemicals were of the highest quality obtainable.

* Abbreviations: MTX, methotrexate (the total number of glutamyl groups appended to the pteroyl moiety of MTX is denoted by the suffix -Glu_n); TS, thymidylate synthase; AT, phosphoribosylaminoimidazolecarboxamide (AICAR) transformylase; DHFR, dihydrofolate reductase; and PG, polyglutamate.

Preparation of reduced folates. $H_2PteGlu_1$ and $H_2PteGlu_5$ were reduced from PteGlu and $PteGlu_5$ as previously described [13, 14] and purified by recrystallization. (6*S*)-Tetrahydrofolate Glu_1 and Glu_5 ($H_4PteGlu$-Glu_5) were prepared by the enzymatic reduction of the corresponding $H_2PteGlu_1$ as previously described [4] and purified by DEAE cellulose chromatography using a linear gradient of ammonium acetate (pH 6.0) from 0.01 to 1.5 M. Peak tubes containing purified tetrahydrofolate were identified by the UV spectra characteristic for tetrahydrofolate. The lack of contamination by oxidized folates or folate metabolites was confirmed by the UV spectra of the purified product and the lack of dihydrofolate reductase active contaminates. The latter test was specific for folic acid and dihydrofolate contamination and was performed using a spectrophotometric assay with *Lactobacillus casei* DHFR, NADPH in 100 mM Tris/HCl buffer, pH 7.2, and the purified folate preparation as the substrate source. 5,10-Methylene tetrahydrofolate Glu_1 and Glu_5 (CH_2-H_4-PteGlu-Glu_5) were prepared prior to each experiment by adding 84 μmol of formaldehyde to 10 μmol of (6*S*)-H_4-PteGlu or Glu_5 to 10 ml of a buffer solution (0.5 ml of 1 M KH_2PO_4, pH 7.2, 10 mg bovine albumin, 9.5 ml water and 14 mM 2-mercaptoethanol).

5-Formyl-$H_4PteGlu_1$ and -Glu_5 were prepared by enzymatic reduction of $H_2PteGlu_1$ or -Glu_5 as described above followed by formylation according to published methods [15, 16]. The 5-formyl-$H_4PteGlu_1$ and -Glu_5 were purified by DEAE-cellulose column chromatography as described above for $H_4PteGlu$, and the peak tubes containing the 5-formyl-$H_4PteGlu_1$ and -Glu_5 were identified by UV spectroscopy. 10-Formyl-$H_4PteGlu_n$ was formed from 5-formyl-$H_4PteGlu_n$ prior to each experiment according to the method of Rabinowitz [17] by first acidifying the 5-formyl-$H_4PteGlu_n$ to pH 1.5 with 0.1 M HCl and allowing the 5-10-methenyl-$H_4PteGlu_n$ to form over 1 hr at room temperature. 10-Formyl-$H_4PteGlu_n$ was then formed by normalizing the pH with 0.1 M KOH. During each experiment, the 10-formyl-$H_4PteGlu_n$ was kept in 1% 2-mercaptoethanol in the dark at 4°. The concentrations of the 10-formyl-$H_4PteGlu_n$ were determined spectrophotometrically by using an extinction coefficient of 22,000 $cm^{-1}\,M^{-1}$ at 258 nm at pH 7.0 [5]. The concentrations of 10-formyl-$H_4PteGlu_n$ were corroborated by spectral measurement of the intermediate 5-10-methenyl-$H_4PteGlu_n$. Concentrations derived by the two measurements differed by < 5%, and no additional UV peaks were detected.

Enzyme source and purification. The ZR-75 human breast cancer cell line was used as the source of TS. The cells were grown in continuous monolayer by the Fermentation Production Facility, Frederick Cancer Research Facility (Frederick, MD) and were stored at −40° until used. TS was purified according to the method of Dolnick and Cheng [18].

A human breast cancer cell line, MCF-7, was used as the source of AT [19]. The cells were grown in continuous monolayer by HEM Laboratories (Rockville, MD) and were stored at −40° until used. AT was purified according to previously described methods [5].

Protein determination. Protein was estimated spectrophotometrically with a Bio-Rad Protein Assay kit using bovine serum albumin as a standard.

TS assay. TS was assayed by a modification of the tritium release procedure of Roberts [20] as previously described [4]. All assays were performed in duplicate. Reaction velocities were found to be linear with time and enzyme concentration under conditions used in these experiments.

AT assay. AT activity was measured spectrophotometrically as described by Black *et al.* [21].

Data analysis. Enzyme kinetics were analyzed by conventional double-reciprocal plots, and the graphic estimates of parameters were used as initial estimates for computerized curve-fitting using a weighted nonlinear least-squares method. We used the program "enzyme," developed by Drs. David Rodbard and Rudy Lutz (National Institute of Child Health and Human Development, Bethesda, MD) for obtaining inhibitory kinetic constants [4].

Results

TS inhibition. We studied the effects of 7-OH-MTX-Glu_1 and 7-OH-MTX-Glu_4 as inhibitors of TS. Inhibition constants (K_i) for each compound were determined at a constant saturating dUMP concentration of 1×10^{-5} M and at variable concentrations of mono- or pentaglutamated folate (Table 1). These results illustrated that, whereas 7-OH-MTX-Glu_1 was a weak inhibitor of TS (K_i 1.7×10^{-5} M), the polyglutamated 7-OH-MTX-Glu_4 showed a 40-fold increase in inhibitory potency (K_i 4.0×10^{-7} M) in the presence of the monoglutamated substrate. The tetraglutamated inhibitor was also found to be greater than 100-fold more potent than the monoglutamated inhibitor when tested with the pentaglutamated substrate.

AT inhibition. We also studied the effects of 7-OH-MTX-Glu_1 and -Glu_4 as inhibitors of AT. Inhibition constants of each compound were determined at a constant AICAR concentration, 1×10^{-5} M, and at variable concentrations of the mono- or pentaglutamated folate (Table 1).

When tested against the monoglutamated substrate (10-formyl-$H_4PteGlu_1$), 7-OH-MTX-Glu_1 was a weak inhibitor of AT (K_i 3.8×10^{-5} M), but the polyglutamated 7-OH-MTX-Glu_4 showed a greater than 1000-fold increase in inhibitory potency (K_i 3.0×10^{-8} M). Against the pentaglutamated substrate (10-formyl-$H_4PteGlu_5$), 7-OH-MTX-Glu_1 was again a poor inhibitor of AT (K_i 1.8×10^{-4} M). As compared to the monoglutamated inhibitor, the polyglutamated inhibitor 7-OH-MTX-Glu_4 showed an almost 500-fold increase in inhibition (K_i 4.0×10^{-7} M) when 10-formyl-$H_4PteGlu_5$ was used as substrate. We observed a consistent reduction in inhibitor potency (5- to 10-fold) when the reactions were conducted using a polyglutamated AT substrate.

Discussion

These studies demonstrate that the major metabolite of MTX, 7-OH-MTX-Glu_1 and its Glu_4 metabolite, inhibited the enzymes of *de novo* pyrimidine and purine synthesis. Since the level of 7-OH-MTX-Glu_1 exceeds that of MTX (*in vivo*) after 10 hr and the $T_{\frac{1}{2}}$ of 7-OH-MTX-Glu_1 is about 3 times longer than the terminal $T_{\frac{1}{2}}$ of MTX, and since 7-OH-MTX undergoes polyglutamation, 7-OH-MTX polyglutamates may play an important role in augmenting and sustaining direct inhibition of TS and AT following MTX therapy. The inhibitory actions of these compounds may help to explain the competitive nature of reduced folate rescue as competitive amounts of the folate substrates would be required to overcome inhibition of enzymes at points in the metabolic pathway other than DHFR if these enzymes were under the direct inhibitory influence of MTX metabolites. The failure of MTX to quantitatively deplete intracellular reduced folates [22] suggests that MTX may exert its cytotoxic effects by virtue of direct inhibition of enzymes by dihydrofolate polyglutamates and/or MTX polyglutamates, rather than through an indirect mechanism of reduced folate depletion. 7-OH-MTX-Glu_1 and its polyglutamates may contribute to and sustain this direct inhibition. To the extent that the polyglutamation of MTX and 7-OH-MTX-Glu_1 is a more active process in tumor cells than in normal tissues [23, 24], this metabolic step may contribute to the selective action of MTX and the selectivity of leucovorin rescue.

The inhibitory capacities of 7-OH-MTX-Glu_1 and -Glu_4 may be compared to those of MTX-Glu_1 and -Glu_4. In general, the 7-OH derivatives were found to be similar in

Table 1. Inhibition of human thymidylate synthase and AICAR transformylase by 7-OH-MTX-Glu_1 and -Glu_4

Folate cofactor	K_m (μM)	V_{max} (pmol/min/mg)	K_i* 7-OH-MTX (μM)	
			Glu_1	Glu_4
TS				
CH_2-H_4PteGlu$_1$	$2.7 \pm 3.0 \times 10^1$	$7.7 \pm 0.6 \times 10^1$	$1.7 \pm 0.4 \times 10^1$	0.4 ± 0.1
CH_2H_4PteGlu$_5$	4 ± 0.1	$3.2 \pm 0.1 \times 10^1$	$3.8 \pm 2.0 \times 10^2$	3 ± 1
AT				
10-Formyl-H_4PteGlu$_1$	$5.4 \pm 1.3 \times 10^1$	$1.7 \pm 0.4 \times 10^1$	$3.8 \pm 1.8 \times 10^1$	0.03 ± 0.01
10-Formyl-H_4PteGlu$_5$	6 ± 1	$1.5 \pm 0.3 \times 10^1$	$1.8 \pm 0.4 \times 10^2$	0.4 ± 0.1

TS was purified 100-fold from human ZR-75 breast cancer cells to a final specific activity of 0.35 nmol/min/mg protein at 37°. The inhibition constants (K_i) for each inhibitor (7-OH-MTX-Glu_1 and 7-OH-MTX-Glu_4) are tabulated with respect to both mono- and pentaglutamated folate cosubstrates. AT was purified 125-fold from human MCF-7 breast cancer cells to a final specific activity of 147 nmol/min/mg protein at 37°. The inhibition constants (K_i) for 7-OH-MTX-Glu_1 and -Glu_4 are tabulated with respect to both mono- and pentaglutamate folate cosubstrates. The Michaelis–Menten constants are also reported for each folate. All parameters were calculated using computerized nonlinear least-squares curve-fitting as described under "Data analysis." Each point represents the mean (± SEM) of two to five independent experiments.

* Based on a competitive model.

potency of inhibition of each enzyme when compared to MTX and MTX-PGs [4, 5]. Further, the somewhat unusual dependency of inhibitory potency on the glutamylated state of the folate substrates has been reported previously for MTX and MTX-PGs [4, 5]. This finding suggests that the interaction of the inhibitors and the folate substrates at the active site of the enzymes is not a simple interaction but one that may involve enzyme binding of the ligands at the level of the polyglutamate tail in addition to the pteridine portion of the substrates and inhibitors. It is beyond the scope of the data presented in this report to postulate a mechanism underlying this complex interaction.

Plasma concentrations of 7-OH-MTX-Glu_1 in patients treated with moderate-dose MTX are in the range of 10^{-6}–10^{-7} M [7]. With K_i values for the inhibition of TS and AT by polyglutamated 7-OH-MTX of less than 1 μM, depending on the state of polyglutamation of the intracellular folates, inhibition of these enzymes may be possible with the levels of 7-OH-MTX-Glu_n that are achieved clinically. Further work in monitoring the levels of 7-OH-MTX-Glu_1 in patients and in determining the extent of 7-OH-MTX PG formation in malignant tissues will be useful in evaluating the contribution of this metabolite to the cytotoxic effects of MTX.

In summary, we examined the potencies of 7-hydroxymethotrexate (7-OH-MTX, 7-OH-MTX-Glu_1) and tetraglutamated 7-hydroxy-MTX (7-OH-MTX-Glu_4), both metabolites of methotrexate (MTX), on the inhibition of thymidylate synthase (TS) and phosphoribosylaminoimidazolecarboxamide (AICAR) transformylase (AT). TS was purified from the ZR-75 human breast cancer cell line to a specific activity of 0.35 nmol/min/mg protein. For TS, the potency of 7-OH-MTX-Glu_1 was similar to that of MTX with an inhibition constant (K_i) of 1.7×10^{-5} M with respect to the monoglutamated folate substrate. The tetraglutamate of 7-OH-MTX-Glu_1 was 40-fold more potent an inhibitor of TS than the monoglutamate, and again its potency was similar to that of MTX-Glu_4 with a K_i of 4.0×10^{-7} M. AT was purified from human MCF-7 breast cancer cells to a specific activity of 147 nmol/min/mg protein. 7-OH-MTX-Glu_1 was found to inhibit the catalytic reaction with a potency similar to MTX with a K_i of 3.8×10^{-5} M. The 7-OH-MTX-Glu_4 was over 1000-fold more potent than the monoglutamated compound with regard to the monoglutamated folate substrate. The potency of inhibition by 7-OH-MTX-Glu_1 and 7-OH-MTX-Glu_4 was dependent on the polyglutamated state of the folate substrates and was diminished when studies using pentaglutamated folates. These findings imply that 7-OH-MTX polyglutamates may augment or sustain direct inhibition of TS and/or AT following MTX therapy. As with MTX-Glu_4, the 7-OH-MTX-Glu_4 was more potent than its parent compound as an inhibitor of both enzymes.

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Interrelationship of 3-deazaguanine-induced growth inhibitory actions in L1210 cells*

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3-Deazaguanine (c^3Gua)† is a guanine analog whose cancer chemotherapeutic properties are currently being investigated clinically [1]. Studies from this laboratory using L1210 leukemic cells *in vitro* have shown that c^3Gua-induced inhibition of DNA synthesis and protein synthesis is closely correlated with the cytotoxic action of the compound [2]. The actions of c^3Gua on DNA and protein synthesis have been associated previously with incorporation of c^3Gua into DNA [2], and with inhibition of initiation of translation [3], respectively. In addition, in L1210 cells, GTP pools decline after c^3G exposure, whereas c^3Gua is anabolized to c^3GTP, which accumulates and is incorporated into RNA [2].

The objective of this study was to assess the roles of various drug effects in cytotoxicity by examining cell recovery following drug removal from the incubation medium. In addition, the possible impairment of translation into protein of mRNA containing c^3Gua was studied.

Materials and methods

[*Methyl*-^{3}H]Thymidine (Thd) (20 Ci/mmol) and [4,5-^{3}H]L-leucine (58 Ci/mmol) were supplied by the New England Nuclear Corp. and ICN Biochemicals, Inc. respectively. [2-^{14}C]c^3Gua mesylate (9.47 mCi/mmol) and c^3Gua mesylate were provided by Dr. R. Jackson, Warner-Lambert Co., Ann Arbor, MI. Additional c^3Gua has been donated previously by ICN Biochemicals, Inc. c^3GTP was made available by Dr. Priscilla Saunders, M. D. Anderson Hospital and Tumor Institute, Houston, TX.

Tissue culture. The L1210 cell line was maintained in RPMI 1640 medium supplemented with 10% dialyzed horse serum (Gibco), 60 μg/ml penicillin G, and 100 μg/ml streptomycin sulfate. Cells were grown under 5% CO_2/air at 37° and were diluted regularly to maintain logarithmic growth.

Assessment of recovery of cells following c^3Gua-induced exposure. Cells (100 ml, 5×10^4 cells/ml) were incubated with 4, 10, or 30 μM c^3Gua for 12 or 24 hr, were washed to remove the drug, resuspended in the original volume of drug-free medium, and reincubated at 37° to permit cell recovery. Two aliquots of 2 ml were taken from each culture at 0, 6, 12, 18, and 24 hr after drug removal and were assayed for [^{3}H]Thd and [^{3}H]leucine incorporation as previously described [2, 4]. In other aliquots, GTP and c^3GTP levels were analyzed by high pressure liquid chromatography [2]. Amounts of c^3GTP in extracts were quantified by comparing area under the curve for the c^3GTP peak to a standard curve derived from cells treated with [^{14}C]c^3Gua. Area under the curve for the UV-absorbing eluate was consistent with the radioactivity data ($r^2 = 0.84$). GTP was quantified similarly from AUC standards.

Assessment of the effect of c^3Gua on viability. L1210 cells were incubated with 0, 4, 10, or 30 μM c^3Gua for 24 hr and then allowed 0, 12, 18, or 24 hr recovery in drug-free medium before plating. Colony formation assays were then performed [5].

RNA isolation and analysis. To study the activity of c^3Gua-containing mRNA in *in vitro* translation, L1210 cultures (5×10^5 cells/ml, 200 ml) were incubated with 0, 4, 10 or 30 μM c^3Gua for 24 hr, RNA was isolated by the method of Chirgwin *et al.* [6] and was then enriched for poly A$^+$ RNA by oligo-deoxythymidylate cellulose chromatography. The amount of poly A$^+$ RNA isolated from 10^8 L1210 cells incubated with 0–30 μM c^3Gua for 24 hr ranged from 16.8 to 21 μg, with A_{260}/A_{280} ratios of 2.00 ± 0.03.

To assess incorporation of ^{14}C-labeled c^3Gua into mRNA, L1210 cells (2×10^5 cells/ml) were incubated with 20 μM [2-^{14}C]c^3Gua (4.41 mCi/mmol) for 24 hr, and RNA was isolated by cesium sulfate density gradient centrifugation [2, 7]. mRNA from these cells prepared as above was collected on pre-wetted Whatman GF/C filters to which 50 μg calf thymus DNA had been added as a carrier. The filters were washed twice with 5 ml of cold 5% tri-

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† Abbreviations: dT, deoxythymidylate; c^3Gua, 3-deazaguanine; c^3GTP, 3-deazaguanosine triphosphate; and Thd, thymidine.