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Synthesis and retention of methotrexate polyglutamates by human small cell lung cancer

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The folic acid analog methotrexate (MTX-Glu_1)* has a broad spectrum of antitumor activity. By inhibiting dihydrofolate reductase (DHFR; EC 1.5.1.3), MTX-Glu_1 restricts repletion of biologically active reduced folate pools and impairs thymidine, purine, and amino acid synthesis. As a single agent in high doses with folinic acid rescue, MTX-Glu_1 has been used in the treatment of osteosarcoma, lymphoma, cancer of the head and neck, and small cell lung cancer (SCLC) [1–3]. By studying the determinants of MTX-Glu_1 sensitivity in cell lines established from patients, more optimum dosing regimens and concepts for improved folate analogs might be devised.

It has been appreciated recently that, like the physiologic folates themselves [4], MTX-Glu_1 can undergo intracellular metabolism to poly-γ-glutamyl derivatives in both normal [5] and malignant [6] cells. Further, there is evidence to suggest that the polyglutamate forms of MTX-Glu_1 (MTX-PGs) are preferentially retained by cells after removal of extracellular drug and can cause prolonged cytotoxicity [7]

We have investigated the metabolism of MTX-Glu_1 to MTX-PGs with high performance liquid chromatography (HPLC) [8] in drug-sensitive and -resistant cell lines isolated from patients with SCLC. One cell line (NCI-H187) was derived from a previously untreated patient, while the second line (NCI-H249) was isolated from a patient in relapse following initial response to single-agent high-dose MTX-Glu_1 with folinic acid (CF) rescue. These studies indicate that this type of human cancer is capable of forming MTX-PG derivatives with up to five glutamyl groups and selectively retains these derivatives for at least 72 hr after removal of extracellular drug. In addition, these retained drug metabolites can cause delayed cytotoxicity in the absence of extracellular MTX-Glu_1.

Materials and methods

Compounds. [3′,5′,9-^{3}H]MTX-Glu_1 (sp. act. 20 Ci/mmole; Amersham, Arlington Heights, IL) was purified by DEAE-cellulose chromatography [9], as was unlabeled drug (Drug Synthesis and Chemistry Branch, NCI, Bethesda, MD). All other chemicals were of reagent grade and purchased from either the Fisher Scientific Co. (Pittsburgh, PA) or the Sigma Chemical Co. (St. Louis, MO). Fetal calf serum (FCS) was obtained from Biofluids, Inc. (Rockville, MD) and dialyzed in Spectrophor tubing (3787-D22) (A. H. Thomas Co., Philadephia, PA) against 8 liters of 0.9% NaCl containing Ag-1-X8 resin (Biorad Laboratories, Richmond, CA) at 4°.

Cell lines. The establishment and characterization of continuous cell lines of human SCLC have been described previously [10]. NCI-H249 was isolated after the patient had received and initially responded to 12 monthly cycles of MTX-Glu_1 at a dose of 1.5 g/m^2 followed by CF rescue. We have previously described this line [11], which possesses unstable amplification of the gene coding for DHFR in association with double-minute chromosomes. The cells are carried in suspension culture with doubling times of 60 hr (NCI-H187) and 68 hr (NCI-H249). The response of NCI-H249 to MTX-Glu_1 and its ability to polyglutamate and retain drug were determined both early in its fifth *in vitro* passage and following 3 months of culture in drug-free medium.

Cytotoxicity studies and assay of intracellular MTX-PGs. To determined the delayed effects of retained drug on survival, cells were incubated at a concentration of 5×10^5/ml for 24 hr in 25-cm^2 plastic flasks (Costar, Cambridge, MA) containing RPMI-1640 with 10% dialyzed FCS, 2 mM L-glutamine, and either 0.2 or 10 μM MTX-Glu_1. The incubation medium was further supplemented with 10 μM thymidine (dT) and 10 μM deoxyinosine (dI); this medium (containing "rescue") allowed normal cell growth in the presence of MTX-Glu_1. At the end of 24 hr, cells were washed three times in iced phosphate-buffered saline (PBS) and resuspended in drug-free medium lacking exogenous dT or dI. At daily intervals for 3 days, cells were counted in a model B Coulter counter and viability was determined by exclusion of trypan blue. Survival was compared to control cells to which no drug had been added. Viability of the control cells was >95%. All experiments were performed in triplicate, and percent control survival was expressed with standard deviation from the mean.

In experiments for intracellular MTX-PG formation, cultures were incubated as above with [^{3}H]MTX-Glu_1 for 24 hr and then washed in iced PBS. A 5-ml aliquot was sampled for determination of intracellular drug profile at the end of the incubation period, and the remaining cells were resuspended in drug-free medium supplemented with dT and dI. After 3 days, cells were harvested, washed, and processed for HPLC determination of intracellular MTX-Glu_1 and MTX-PGs as has been described previously [12]. Drug levels are expressed as nmoles MTX/mg protein as determined by the method of Lowry *et al.* [13].

Results

Intracellular MTX and MTX-PGs. Table 1 describes the intracellular accumulation and retention of MTX-Glu_1 and MTX-PGs during a 24-hr drug incubation followed by a 3-day efflux period in drug-free medium. Measured intracellular binding was determined from the drug efflux plateau as previously described [11]. Exposure to 0.2 μM [^{3}H]MTX-Glu_1 for 24 hr was insufficient to saturate the intracellular binding capacity in either cell line. In the absence of free drug, only small amounts of MTX-PGs were formed—predominantly MTX-Glu_2 and small amounts of MTX-Glu_3 and -Glu_4. Neither cell line synthesized MTX-Glu_5 under these conditions.

Following a 24-hr incubation in 10 μM [^{3}H]MTX-Glu_1, NCI-H187 accumulated 41.48 nmoles drug/g protein, far in excess of the measured intracellular binding capacity (3.10 nmoles/g protein). Forty-nine percent of the intracellular drug (20.2 nmoles/g) present at the end of incubation was in the form of MTX-PGs. During subsequent efflux in drug-free medium, polyglutamates were selectively retained to become the predominant intracellular form of drug. Total drug retention at 72 hr (4.20 nmoles/g) remained sufficient to exceed pretreatment drug-binding capacity over the entire observation period.

* Abbreviations used: MTX-Glu_1, methotrexate (4-amino-10-methylpteroylglutamic acid); and MTX-Glu_n, methotrexate polyglutamate (4-amino-10-methylpteroylglutamyl-γ-glutamyl), where n indicates the total number of glutamate residues in the molecule.

In the early, drug-resistant passage of NCI-H249, a 24-hr incubation in 10 μM MTX-Glu_1 was sufficient initially to saturate the amplified intracellular binding capacity (34.7 nmoles drug/g), with 130 nmoles/g of intracellular drug, 53% of which was present as MTX-PGs. However, subsequent drug retention in drug-free medium was inadequate for prolonged target enzyme saturation. Total intracellular drug fell below intracellular binding capacity to 24.3 nmoles/g after 24 hr of efflux and to 12.8 nmoles/g after 72 hr, with 71% of residual intracellular drug present in polyglutamate form.

In the late, drug-sensitive passage of NCI-H249, a 24-hr incubation in 10 μM MTX-Glu_1 was again adequate to saturate intracellular binding capacity (6.9 nmoles/g) with 114.5 nmoles of intracellular drug/g protein; 68% of intracellular drug was present as MTX-PGs. Subsequent retention of intracellular drug during efflux was similar to the early passage cells, with 12.34 nmoles/g (87% MTX-PGs) retained after 72 hr efflux, a level sufficient to exceed the pretreatment binding capacity for the entire observation period.

Polyglutamate derivatives of higher molecular weight were preferentially retained (Table 1). During the 72 hr of efflux, only 4.4 to 6.6% of parent drug initially present after incubation was retained, while 49 to 90% of synthesized MTX-Glu_5 was conserved.

Cytotoxicity. Cytotoxicity studies established considerable differences between the cell lines. Figure 1B shows the effect of a 24-hr exposure to 10 μM MTX-Glu_1 in both NCI-H187 and the early passage NCI-H249. No cytotoxicity occurred during the incubation period itself, as cells were rescued from MTX-Glu_1 by exogenous dT and dI. However, drug accumulated and was metabolized to MTX-PGs during this period. After cells were washed and resuspended in medium lacking dI and dT, the delayed cytotoxic effect of retained drug was observed in NCI-H187; survival fell to 24% of control after 3 days in drug-free medium. No significant toxicity was observed in the early passage of NCI-H249 after a 10 μM MTX-Glu_1 exposure, or in either cell line after exposure to 0.2 μM drug (Fig. 1A).

However, following 3 months of culture in drug-free medium DHFR levels and drug binding capacity in NCI-H249 approached that of the MTX-Glu_1-sensitive NCI-H187 [11]. Cytotoxicity studies were then repeated as shown in Fig. 1C. A 24-hr exposure to 10 μM MTX-Glu_1 plus rescue now resulted in significant delayed cell kill, with 30% control survival after 3 days in drug-free medium.

Discussion

Intracellular MTX binding capacity and DHFR specific activity. The enzymatic characterization of NCI-H187 and NCI-H249 is presented in Ref. 11. The latter cell line was studied both early in its fifth *in vitro* passage and after 3 months of culture in drug-free medium. There was an excellent correlation between intracellular MTX binding capacity (the efflux plateau reached in each cell line when incubated in drug-free medium following drug exposure) and the specific activity of DHFR measured directly on

Table 1. Formation and retention of intracellular MTX-PGs by human SCLC cell lines

Cell line	Intracellular drug (nmoles/g protein)					
	Total	MTX	MTX-Glu_2	MTX-Glu_3	MTX-Glu_4	MTX-Glu_5
NCI-H187 (Measured intracellular drug-binding capacity = 3.1 nmoles/g protein)*						
0.2 μM MTX						
After 24-hr incubation	1.22	0.78	0.25	0.16	0.03	
After 72-hr efflux	0.29	0.08	0.07	0.10	0.03	
(% retained)	(23.7)	(10.2)	(28)	(62.5)	(100)	
10 μM MTX						
After 24-hr incubation	41.48	21.10	20.20	7.90	1.90	0.20
After 72-hr efflux	4.20	1.40	0.53	1.48	0.61	0.18
(% retained)	(10)	(6.6)	(5.2)	(18.7)	(32)	(90)
NCI-H249 (Measured intracellular drug-binding capacity = 34.7 nmoles/g protein) (early passage)						
0.2 μM MTX						
After 24-hr incubation	4.33	1.93	1.43	0.83	0.14	
After 72-hr efflux	1.86	0.67	0.65	0.46	0.08	
(% retained)	(42.9)	(34.9)	(45.4)	(55.4)	(57.1)	
10 μM MTX						
After 24-hr incubation	130.00	60.97	6.63	36.66	20.80	4.94
After 72-hr efflux	12.80	3.65	0	2.66	4.06	2.43
(% retained)	(9.8)	(5.9)	(0)	(7.2)	(19.5)	(49.2)
NCI-H249 (Measured intracellular drug-binding capacity = 6.9 nmoles/g protein) (late passage)						
10 μM MTX						
After 24-hr incubation	114.50	36.53	15.11	36.30	21.98	4.58
After 72-hr efflux	12.34	1.61	0.21	2.99	5.03	2.48
(% retained)	(10.7)	(4.4)	(1.4)	(8.2)	(22.8)	(54.1)

* Represents drug efflux plateau in each line.

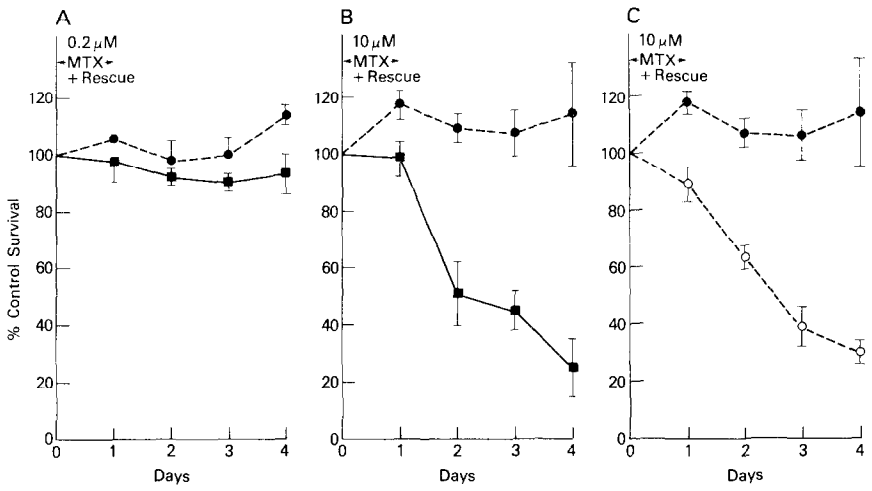

Fig. 1. (A) Cytoxicity of a 24-hr incubation in 0.2 μM MTX for NCI-H187 (■——■) and early passage NCI-H249 (●– – –●). (B) Cytotoxicity of a 24-hr incubation in 10 μM MTX for NCI-H187 (■——■) and early passage NCI-H249 (●– – –●). (C) Cytotoxicity of a 24-hr incubation in 10 μM MTX for early (●– – –●) and late (○– – –○) passage NCI-H249.

cytosol preparations [11]. This suggests that residual intracellular drug after efflux is predominantly bound to target enzyme. During the periods of drug exposure to influx plateau followed by drug efflux, polyglutamate formation was not detectable, so that measured binding capacity represents monoglutamate MTX-Glu_1 bound to DHFR.

An interesting peculiarity of DHFR amplification in NCI-H249 is that it is unstable. The resistant phenotype is associated with the presence of double-minute chromosomes which are lost along with enzyme amplification during culture in drug-free medium [11]. After 3 months of serial passage, target enzyme levels fell by 88% (from 17.6 to 2.04 nmoles NADPH converted/mg protein/min) with a corresponding 80% reduction in intracellular binding capacity (from 34.7 to 6.9 nmoles MTX bound/mg protein). We have observed no change in NCI-H187 DHFR specific activity or MTX binding capacity after 1 year of *in vitro* passage.

In this study we have investigated the determinants of MTX-Glu_1 cytotoxicity in drug-sensitive and -resistant cell lines of human SCLC. The critical requirement for MTX-Glu_1 cell kill is prolonged maintenance of intracellular drug above binding capacity. It has been demonstrated previously that inhibition of DHFR by MTX requires the presence of free intracellular drug [14]. In the absence of free MTX, physiologic folates can compete for binding sites on DHFR, reversing enzyme inhibition. It is also apparent that free intracellular drug is required for synthesis of MTX polyglutamates. Following exposure to low (0.2 μM) drug concentrations, intracellular binding capacity was not saturated and only small amounts of lower molecular weight MTX-PGs were formed.

Even after exposure to cytotoxic concentrations of drug and initial saturation of intracellular binding sites, MTX-Glu_1 itself was poorly retained. In these SCLC cell lines, only 4.4 to 6.6% of MTX-Glu_1 present at the end of a 10 μM drug incubation was retained when cells were incubated in drug-free medium for 3 days (Table 1). However, metabolism of MTX-Glu_1 to more avidly retained polyglutamate derivatives allows the formation of a drug reservoir capable of prolonged enzyme inhibition in the absence of extracellular MTX-Glu_1. These human SCLC cell lines are capable of synthesizing MTX-PGs with two to five γ-glutamyl groups (MTX-Glu_{2-5}). Of these metabolites, MTX-Glu_{4-5} are best retained when cells are incubated in drug-free medium for 3 days. Between 19.5 and 32% of MTX-Glu_4 initially formed was retained after efflux, while 49 to 90% of MTX-Glu_5 was conserved. Although studying retention profiles after 72 hr of efflux might minimize observed differences in drug and metabolite loss, this time point was selected because of the long doubling time of these cells (60–68 hr).

These results are similar to those of Kennedy *et al.* [15], who studied MTX-Glu_1 polyglutamation using gel filtration techniques in cultured human breast cancer cells. Following a 48-hr incubation in 10 μM MTX-Glu_1, cells were incubated in drug-free medium for 48 hr. In these experiments, <5% of intracellular parent drug was retained, while 10% of total MTX-Glu_2 plus MTX-Glu_3 and 30% of MTX-Glu_4–MTX-Glu_6 were conserved. However, total drug retention was not correlated with intracellular binding capacity or cytotoxic effects. Similarly, selective retention of MTX-PGs has been observed in human fibroblasts [5], rat hepatoma cells [16], and Ehrlich ascites cells [6]: however, these previous studies did not determine specifically which polyglutamate derivatives had been retained.

Using human breast cancer cell lines, Jolivet *et al.* [12] have also confirmed preferential retention of MTX-Glu_{4-5} in the absence of extracellular drug. Moreover, inhibition of deoxyuridine incorporation into DNA and inhibition of cell growth could be demonstrated 24 hr after removal of extracellular MTX only in those lines in which drug was retained above binding capacity.

We have now shown that selective retention of high molecular weight MTX-PGs is crucial for the prolonged (>72 hr) saturation of intracellular binding capacity needed for MTX cytotoxicity. In this respect, the MTX-Glu_1-resistant and -sensitive passages of NCI-H249 are illustrative. Following a 24-hr exposure to 10 μM MTX-Glu_1, drug uptake, polyglutamation, and retention were essentially identical in both cell passages. However, in the early passage line, drug retention was insufficient to saturate the amplified binding capacity, and no cytotoxicity was observed (Fig. 1B). After serial culture in drug-free medium resulted in lower target enzyme levels (Table 1), the same uptake and retention profile allowed prolonged enzyme saturation and cell kill (Fig. 1C). Thus, prolonged drug retention appears more dependent on polyglutamate formation than binding capacity.

However, it is known that exposure to MTX-Glu_1 may, in itself, increase intracellular target enzyme levels. This phenomenon has been observed in both human and gene-amplified murine cells and has been ascribed to either enzyme stabilization by drug [17] or rapid increases in

protein synthesis [18, 19]. Thus, verification of enzyme saturation after prolonged drug exposure will require direct measurement of bound and free intracellular drug levels.

In summary, human SCLC cell lines are able to synthesize MTX polyglutamate derivatives with two to five additional γ-glutamyl groups. As has been reported in human breast cancer cell lines [11, 15], higher molecular weight derivatives with more than four glutamyl groups were selectively retained in the absence of extracellular drug, while MTX-Glu_1 and lower molecular weight polyglutamates effluxed rapidly. Polyglutamation of MTX is an important determinant of cytotoxicity, since prolonged drug retention above intracellular binding capacity was necessary for cytotoxic effect.

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Inhibition of mouse lymphocyte 5′-nucleotidase by cannabinoids: a physical toxicity effect

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Cannabinoids have very high partition coefficients for distribution between non-aqueous and aqueous phases. Their affinity for cell membranes results in the inhibition of a number of plasma membrane-bound enzymes when the drugs are present at relatively low concentrations in the aqueous phase. Among the membrane-bound enzymes inhibited are (Na^+–K^+)ATPases (EC 3.6.1.3) of mouse brain synaptosomes [1], human and rat erythrocytes, rat liver mitochondria [2] and rat ileum [3]. Other membrane enzymes inhibited include rat brain and heart mitochondrial NADH dehydrogenases [4], mouse neuroblastoma adenyl cyclase [5], mouse brain synaptosome and mouse lymphocyte acyl coenzyme A:lysophosphatidylcholine acyl transferase (LPC acyl transferase, EC 2.3.1.23) [6], and mouse lymphocyte acyl coenzyme A:lysophosphatidate acyl transferase (LPA acyl transferase) [7]. The ectoenzyme 5′-nucleotidase (EC 3.1.3.5) is present on the outer surface of mammalian plasma membranes, and the mouse lymphocyte enzyme can be inhibited by large hydrocarbons [8]. The widespread occurrence of this membrane enzyme suggests that it could be a useful model for the study of the inhibition of membrane-bound enzymes. In this study we have investigated the inhibition of 5′-nucleotidase by cannabinoids and other hydrocarbons to determine whether such inhibition is consistent with physical toxicity or if there are specific stereochemical requirements for inhibition.

Lymphocyte suspensions were prepared from spleens of 6 to 8-week-old male Swiss–Webster mice. The spleens were gently homogenized with a Teflon–glass homogenizer in 10 ml of cold Hanks' balanced salt solution (HBSS), and connective tissue was removed by filtering the homogenate through a fine mesh brass screen. The filtrate was centrifuged at 4° for 10 min at 280 *g*. Erythrocytes were removed according to the method of Boyle [9]. The resulting cell pellet was resuspended in 10 ml HBSS, and adherent cells were removed by passage through a short presoaked column of glass wool. The filtrate was centrifuged for 10 min at 280 *g* and 4°. This final pellet of lymphocytes was resuspended in RPMI 1640 medium with 25 mM HEPES (*N*-2-hydroxyethyl-piperazine-*N*′-2-ethanesulfonic acid) at