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5-Fluorouracil–methotrexate synergy: Enhancement of 5-fluorodeoxyuridylate binding to thymidylate synthase by dihydropteroylpolyglutamates

(pteroylpolyglutamates/ternary enzyme complexes/combination chemotherapy)

DANIEL J. FERNANDES AND JOSEPH R. BERTINO

Departments of Pharmacology and Medicine, Yale University School of Medicine, New Haven, Connecticut 06510

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ABSTRACT **Ternary complex formation of thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45), 5-fluorodeoxyuridylate (FdUMP), and poly(γ-glutamyl) conjugates of pteroate and methotrexate (MTX) has been examined as a basis for the sequence-dependent synergism of the 5-fluorouracil–MTX combination in inhibiting viability of L1210 murine tumor cells. A 1.4-log (25-fold) increase in the inhibition of soft agar colony formation was observed when MTX preceded 5-fluorouracil, as compared to the reverse sequence. L1210 cells converted 39% of the total intracellular MTX into MTX poly(γ-glutamate)s within 4 hr of exposure to 1 μM MTX. MTX and MTX(γ-glutamate) formed reversible ternary complexes with FdUMP on one site of thymidylate synthase, whereas with 7,8-dihydropteroylpentaglutamate and *l*-5,10-methylenetetrahydropteroylpentaglutamate stoichiometric binding of FdUMP to two sites on thymidylate synthase was observed. The dissociation constants for FdUMP in the ternary complexes formed in the presence of MTX, MTX(γ-glutamate), 7,8-dihydropteroylpentaglutamate, and *l*-5-10-methylenetetrahydropteroylpentaglutamate were estimated to be 370, 27, <10, and <10 nM, respectively, by equilibrium dialysis. We propose that the sequence-dependent effect of MTX plus 5-fluorouracil on L1210 cell viability results from MTX and MTX polyglutamate inhibition of dihydrofolate reductase (tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) and consequently a trapping of intracellular folates as dihydropteroylpolyglutamates, which increase the extent of FdUMP binding to thymidylate synthase.**

The antitumor drug 5-fluorouracil (FUra) is converted intracellularly to FdUMP, which in the presence of 5,10-methylene-5,6,7,8-tetrahydropteroylglutamate (5,10-CH_2-H_4PteGlu) is a tight-binding inhibitor of thymidylate synthase (5,10-methylenetetrahydrofolate:dUMP *C*-methyltransferase, EC 2.1.1.45) (1, 2). The antifolate methotrexate (MTX), a tight-binding inhibitor of dihydrofolate reductase (tetrahydrofolate dehydrogenase; 5,6,7,8-tetrahydrofolate:NADP$^+$ oxidoreductase, EC 1.5.1.3) (3), also enhances binding of FdUMP to *Lactobacillus casei* thymidylate synthase, although not as effectively as does 5,10-CH_2-H_4PteGlu (1). However, folates (4–6) and MTX (7–11) exist in most cells predominantly as poly(γ-glutamyl) conjugates. Recently Washtien and Santi (12) have reported that the ternary complex isolated from L1210 murine tumor cells was approximately 1000 daltons higher in molecular mass than the complex formed by incubation of FdUMP, 5,10-CH_2-H_4PteGlu, and cell cytosol, which suggested the presence of a pteroylpolyglutamate in the ternary complex formed intracellularly.

Accordingly, we have attempted to characterize ternary complexes of reduced pteroyl and MTX polyglutamates with FdUMP and purified L1210 thymidylate synthase because of (*i*) the preponderance of reduced pteroyl and MTX polyglutamates in mammalian cells and their probable functions as the active cofactors (13, 14) or inhibitors (15, 16) of thymidylate biosynthesis *in vivo* and (*ii*) the possible contribution of the formation of ternary complexes to the synergistic antitumor effect observed when FUra and MTX are used in combination cancer chemotherapy. This synergistic effect has been noted only when either tumor-bearing mice (17, 18) or tumor cells in culture (19) are exposed to MTX prior to FUra.

MATERIALS AND METHODS

Materials. MTX (Na salt) was obtained from the Division of Cancer Treatment, National Cancer Institute. MTX(Glu_1) was the generous gift of Samuel Jacobs, University of Pittsburgh School of Medicine. MTX(Glu_2) and MTX(Glu_3) were kindly provided by Charles Baugh, Univeristy of Alabama School of Medicine. FUra was obtained from Hoffmann–La Roche. FdUMP and dUMP (Na salts), Triton X-100, and dithiothreitol were purchased from Sigma. [5-^{3}H]dUMP was purchased from Amersham and had an estimated radiochemical purity of 95% by anion-exchange high-performance liquid chromatography (HPLC). [6-^{3}H]FdUMP was obtained from Moravek Biochemicals (City of Industry, CA); radiochemical purity of various batches was estimated to be between 89% and 96% by anion-exchange HPLC, and specific radioactivity between 12.5 and 16.0 Ci/mmol (1 Ci = 3.7×10^{10} becquerels). *dl*-H_4PteL-Glu prepared (20) and purified (21) as described was obtained from John McGuire, Department of Pharmacology, Yale University School of Medicine. $H_2PteGlu_5$ prepared by sodium dithionite reduction of $PteGlu_5$ and purified on DEAE-Sephadex (22) was kindly provided by James Coward and A. R. Cashmore, Department of Pharmacology, Yale University, and was desalted by Bio-Gel P-2 chromatography for use in equilibrium dialysis. $H_2PteGlu_5$ was converted to *l*-$H_4PteGlu_5$ by reaction catalyzed by dihydrofolate reductase (22). The concentrations of $H_2PteGlu_5$ stock solutions were calculated

Abbreviations: HPLC, high-performance liquid chromatography; FUra, 5-fluorouracil; FdUMP, 5-fluorodeoxyuridylate; MTX, methotrexate, 2,4-diamino-10-methylpteroylglutamate; MTX(Glu_1), methotrexate(γ-glutamate), 2,4-diamino-10-methylpteroyldiglutamate; MTX(Glu_2), methotrexate(γ-glutamate-γ-glutamate), 2,4-diamino-10-methylpteroyltriglutamate; $H_2PteGlu$, 7,8-dihydropteroylglutamate; $H_2PteGlu_5$, 7,8-dihydropteroylpentaglutamate; 5,10-CH_2-H_4PteGlu, *dl*-5,10-methylene-5,6,7,8-tetrahydropteroylglutamate, racemic mixture of the physiologically active diastereoisomer (*l*) and the nonphysiological diastereoisomer (*d*); 5,10-CH_2-$H_4PteGlu_5$, *l*-5,10-methylene-5,6,7,8-tetrahydropteroylpentaglutamate. $PteGlu_n$ is used as the general term for polyglutamyl conjugates of pteroic acid or any mixture of them. All glutamic acid residues have the L absolute configuration and are linked in peptide bonds through the γ-carboxyl groups in MTX and pteroylpolyglutamates.

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spectrophotometrically. The purity of $H_2PteGlu_5$ and *l*-$H_4PteGlu_5$ was checked on reversed-phase HPLC (23). No contamination of $H_2PteGlu_5$ with either $H_4PteGlu_5$ or 5,10-CH_2-$H_4PteGlu_5$ was detected either spectrally or by reversed-phase HPLC.

The composition of buffers was as follows: buffer A, 50 mM Tris·HCl, pH 7.5/10% (vol/vol) glycerol/2 mM dithiothreitol; buffer B, 250 mM Tris·HCl, pH 7.5/10% glycerol/13 mM 2-mercaptoethanol/6 mM formaldehyde; buffer C, 50 mM Tris·HCl, pH 7.5/10% glycerol/20 mM 2-mercaptoethanol/3 mM formaldehyde/42 mM NaCl; buffer D, 50 mM Tris·HCl, pH 7.5/10% glycerol/20 mM 2-mercaptoethanol/168 mM NaCl. The concentrations of all drugs and folates were determined spectrophotometrically.

Soft Agar Cloning. The viability of logarithmically growing L1210 cells (doubling time 10.8 hr) after drug exposure was assayed by cloning in soft agar as described by Chu and Fischer (24) with the following modification. Because of the large kill produced by combination treatment it was sometimes necessary to clone between 10^4 and 10^6 cells per tube. However, the cloning efficiency of untreated cells was found to decrease when they were cloned in the presence of greater than 10^4 drug-treated cells. Thus, as an attempt to avoid an overestimation of cell kill, untreated cells were added to parallel groups of treated cells and cloned. The cloning efficiency of untreated cells in the presence of drug-treated cells (corrected cloning efficiency) was calculated according to the following formula:

$$\text{Corrected cloning efficiency } (E) = \frac{C_{t+u} - C_t}{N_u},$$

in which C_{t+u} is the number of clones formed in tubes containing both treated and untreated cells, C_t is the number of clones formed in tubes containing only treated cells, and N_u is the number of untreated cells cloned. Cell viability was then calculated according to the formula:

$$\text{Cell viability } (\%) = \frac{C_t \times 100}{N_t \times E},$$

in which N_t is the number of treated cells cloned.

Enzyme Purification. L1210 thymidylate synthase was purified by 10-formyl-5,8-dideazafolate affinity chromatography according to the method of Rode *et al.* (25). L1210 cells propagated intraperitoneally in CDF_1 mice were used as a cell source. The electrophoretically homogeneous preparation had a specific activity of 383 nmol of thymidylate synthesized per min per mg of protein. Protein concentrations were estimated by the Coomassie blue staining method (26).

Enzyme Assays. Thymidylate synthase was assayed by the tritium displacement method of Lomax and Greenberg (27) as modified by Dolnick and Cheng (28) in a final volume of either 40 or 105 μl. The reaction mixture was incubated for 2 min at 37°C before the initiation of the reaction by the addition of enzyme. Tritium release was linear with respect to time and protein concentration under the conditions used in all experiments. One enzyme unit is defined as the quantity of enzyme required to form 1 nmol of dTMP per min at 37°C under our assay conditions. Enzyme purity was estimated by comparison of the specific activity of various partially purified preparations to that of the homogeneous preparation. Enzyme concentrations were calculated by titration of thymidylate synthase with FdUMP in the presence of saturating 5,10-CH_2-$H_4PteGlu$.

Quantitation of Intracellular MTX and MTX Polyglutamates. The intracellular concentrations of MTX and MTX polyglutamates were determined as described (8, 29).

Equilibrium Dialysis. A Hoefer Scientific Instruments microdialyzer equipped with 250-μl dialysis chambers was used. Membranes having a 12,000–14,000 molecular weight cut-off were prepared by boiling for 5 min in 5 mM Na_2EDTA plus 16 mM $NaHCO_3$. With the exception of enzyme, equimolar amounts of all compounds were added to both sides of the membrane in buffer A containing 0.1% (vol/vol) Triton X-100, which served to stabilize the enzyme activity (25). Dialysis was carried out at 4°C for 24 hr at which time the [^{3}H]-FdUMP had equilibrated across the membrane. No loss of enzyme activity was detected during dialysis of control samples lacking FdUMP. After 24 hr the entire contents of each chamber was removed with a syringe and combined with the volume obtained after two successive washings of each chamber with 200 μl of buffer A. Radioactivity was measured as described (8). After this procedure no more than 2% of the recovered radioactivity remained associated with the membrane over the entire range of [^{3}H]FdUMP concentrations employed. Because of the low enzyme concentrations used, no corrections were made for Donnan effects. The amount of bound ligand was calculated as the difference in radioactivity between the protein and ligand sides of the chamber. Scatchard analysis (30) of the data was performed by plotting $(\bar{V}/[L_f]) = (\bar{V}/K_d) + (n/K_d)$, in which $\bar{V}$ is the moles of ligand bound per mole of protein, $[L_f]$ is the free ligand concentration, n is the number of moles of ligand binding sites per mole of protein, and K_d is the intrinsic ligand dissociation constant of a binding site. Data points were fitted by eye.

RESULTS

Effect of FUra and MTX on Cell Viability. It was observed that, when L1210 cells were exposed to both FUra and MTX, the effect on L1210 cell viability was highly dependent upon the sequence of drug administration (Table 1). The combined inhibition of colony formation by a 4-hr exposure to 10 μM FUra followed by a 4-hr exposure of 1 μM MTX (3.7-log cell kill) was less than the theoretical additive log cell kill of 4.5 expected with FUra (1.7-log kill) plus MTX (2.8-log kill). On the other hand, when cells were exposed to MTX prior to FUra, a 5.1-log reduction in tumor cell viability was seen, which was greater than the sum of the FUra and MTX effects. Simultaneous FUra plus MTX produced an additive response. This study was repeated two additional times with similar results.

The proposed relationship between FUra and MTX metabolism and thymidylate synthase ternary complex formation is illustrated in Fig. 1. We postulated that the sequence-dependent effect of FUra and MTX on cell viability was related to inhibition of thymidylate synthase via formation of ternary enzyme complexes. It was reasoned that exposure of L1210 cells to MTX before FUra produced a synergistic cell kill because it resulted in either (*i*) the synthesis and accumulation of MTX polyglutamates prior to the FUra exposure, which subsequently could

Table 1. Sequence-dependent effects of MTX and FUra on L1210 cell viability

Condition	log(cell kill)*
FUra	1.7 ± 0.05
MTX	2.8 ± 0.34
FUra → MTX	3.7 ± 0.14
MTX + FUra	4.7 ± 0.24
MTX → FUra	5.1 ± 0.25

L1210 cells grown at a density of 2×10^5 cells per ml were exposed to either 1 μM MTX or 10 μM FUra. Each drug exposure either alone or in combination was for 4 hr. Cells were washed once in drug-free medium after each 4-hr exposure and cloned in soft agar, and cell viability was determined. Cloning efficiency of untreated cells was 89%.

* Mean ± SD for four measurements.

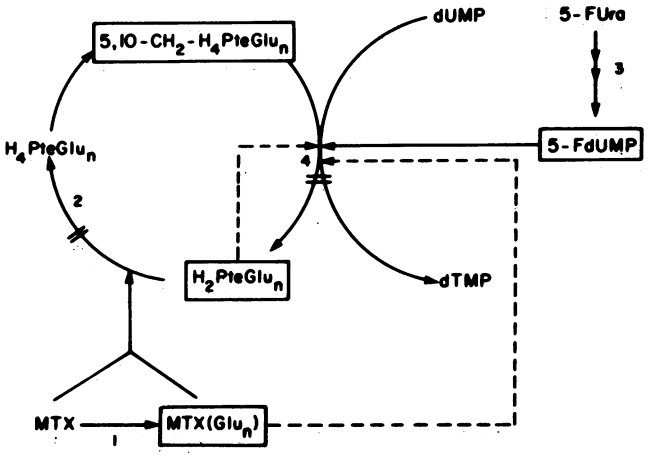

FIG. 1. Relationship among MTX, FUra, and thymidylate synthase. MTX is converted by folylpolyglutamate synthetase (reaction 1) to MTX poly(γ-glutamate)s. Both MTX and MTX(Glu_n), by inhibiting dihydrofolate reductase (reaction 2), block the generation of tetrahydropteroylpolyglutamates, which results in the accumulation of dihydropteroylpolyglutamates. FUra is anabolized along the pathways available for uracil to FdUMP (reactions 3), which in the presence of 5,10-CH_2-$H_4PteGlu_n$ is a tight-binding inhibitor of thymidylate synthase (reaction 4). MTX(Glu_n) and $H_2PteGlu_n$ may also form ternary complexes with FdUMP and thymidylate synthase.

stimulate tight binding of FdUMP to thymidylate synthase; or (*ii*) inhibition of dihydrofolate reductase and consequently an increase in dihydrofolates that also could enhance FdUMP binding to thymidylate synthase.

Biosynthesis of MTX Polyglutamates. In order to determine if MTX polyglutamates were formed intracellularly during the 4-hr exposure to MTX prior to FUra, L1210 cells were incubated with 1 μM MTX for 4 hr and the cell extracts were analyzed by HPLC. L1210 cells converted 39% of the total intracellular MTX into MTX poly(γ-glutamate)s within 4 hr (Table 2). The intracellular concentrations of MTX, MTX(Glu_1), and MTX(Glu_2) were 2.4, 0.4, and 1.0 μM, respectively.

Effects of Reduced Pteroylpolyglutamates on Tight-Binding Inhibition of Thymidylate Synthase. Because the predominant folate forms in most mammalian cells are pentaglutamates (4, 6), it appeared important to examine the effect of 5,10-CH_2-$H_4PteGlu_5$ and $H_2PteGlu_5$ on FdUMP binding to thymidylate synthase. The dihydro compound was of particular interest, because its intracellular concentration is thought to increase markedly after cells are exposed to MTX (31–33).

Table 2. Distribution of MTX and MTX polyglutamates

Radiolabeled species	Amount, pmol/10^7 cells*	Conc., μM	% total radioactivity
p-Aminobenzyl-Glu_n, pteridin-6-COOH, and ^3H_2O	0.8 ± 1.0	0.1	3
Unidentified products	0.4 ± 0.4	0.05	1
MTX	16.3 ± 1.8	2.4	57
MTX(Glu_1)	3.0 ± 0.5	0.4	10
MTX(Glu_2)	6.7 ± 0.7	1.0	23
MTX(Glu_3)	1.5 ± 0.2	0.2	5
Total MTX polyglutamates [MTX(Glu_{1-3})]	11.1	1.6	39

L1210 cells grown at a density of 1×10^5 cells per ml were incubated for 4 hr with 1 μM [^{3}H]MTX (final specific radioactivity of 0.73 Ci/mmol) in a volume of 25 ml. The cells were washed twice with drug-free medium at 4°C, and the ^{3}H-labeled compounds were extracted by boiling for 10 min in 0.1 M sodium phosphate, pH 5.5. The extract was centrifuged at 27,000 × *g* for 30 min at 4°C and the supernatant was analyzed by HPLC. The intracellular concentration of each radiolabeled species was calculated by assuming 0.68 ml of water per 10^9 cells (31).

* Mean ± SD for three measurements.

L1210 thymidylate synthase was preincubated with inhibitors, and samples were removed and assayed for enzyme activity in a 1:21 dilution of assay mix. Under these conditions only tight-binding inhibition was measured, because weak complexes would dissociate in the assay and thereby have little effect on enzyme activity. For instance, 20 nM FdUMP preincubated with enzyme in the absence of cofactor was diluted so that its final concentration of 1 nM was insufficient for it to react with 5,10-CH_2-$H_4PteGlu$ in the assay mix to produce inhibition of thymidylate synthase (Table 3). Likewise, neither the natural (*l*) isomer of 5,10-CH_2-$H_4PteGlu_5$ at 10 μM nor $H_2PteGlu_5$ alone at 18 μM produced any statistically significant inhibition of thymidylate synthase activity. However, preincubation of the enzyme with 5,10-CH_2-$H_2PteGlu_5$ (10 μM) and FdUMP resulted in a nearly complete loss of activity. $H_2PteGlu_5$ at 18 μM also increased the affinity of FdUMP to thymidylate synthase. The maximal effect of $H_2PteGlu_5$ could not be tested with this assay, because $H_2PteGlu_5$ alone at concentrations greater than 18 μM resulted in marked inhibition of enzyme activity.

Ternary Complexes of Thymidylate Synthase as Measured by Equilibrium Dialysis. Equilibrium dialysis was performed with purified L1210 thymidylate synthase and variable concentrations of [^{3}H]FdUMP in the presence of MTX, MTX(Glu_1), $H_2PteGlu_5$, or 5,10-CH_2-$H_4PteGlu_5$. Scatchard analysis of equilibrium binding data revealed that in the presence of 40 μM MTX or 10 μM MTX(Glu_1), one mole of FdUMP was bound per mole of thymidylate synthase, as indicated by the intercepts on the abscissa (Fig. 2*A*). However, binding of FdUMP to thymidylate synthase was tighter in the presence of MTX(Glu_1) than in the presence of MTX, with dissociation constants for FdUMP in the ternary complexes estimated at 27 and 370 nM, respectively. In the absence of cofactor, no significant difference in radioactivity between the protein and ligand sides of the chamber was detectable.

FdUMP binding to thymidylate synthase in the presence of reduced pteroylpolyglutamates differed from that with MTX and MTX(Glu_1). In contrast to the reversible single-site binding observed with the antifolates, 10 μM 5,10-CH_2-$H_4PteGlu_5$ and 2 μM $H_2PteGlu_5$ stimulated binding of FdUMP to two sites on

Table 3. Effect of pteroylpolyglutamates on tight-binding inhibition of thymidylate synthase by FdUMP

Reactants in preincubation	Enzyme activity, units/ml*	Activity, %
E	1.46 ± 0.48	100
E + FdUMP	1.41 ± 0.45	97
E + *l*-5,10-CH_2-$H_4PteGlu_5$ (10 μM)	1.41 ± 0.77	97
E + *l*-5,10-CH_2-$H_4PteGlu_5$ (5 μM) + FdUMP	0.91 ± 0.23	62
E + *l*-5,10-CH_2-$H_4PteGlu$ (10 μM) + FdUMP	0.37 ± 0.22	25
E + 7,8-$H_2PteGlu_5$ (18 μM)	1.17 ± 0.27	80
E + 7,8-$H_2PteGlu_5$ (9 μM) + FdUMP	1.12 ± 0.15	77
E + 7,8-$H_2PteGlu_5$ (18 μM) + FdUMP	0.72 ± 0.01	49

Thymidylate synthase (E) at a concentration of 18 nM (specific activity 55 units/mg of protein) was preincubated for 10 min at 37°C with either 5,10-CH_2-$H_4PteGlu_5$ in buffer C or 7,8-$H_2PteGlu_5$ in buffer D in the presence or absence of 20 nM FdUMP. Five-microliter samples were removed and assayed for 45 min at 37°C in a volume of 105 μl (see text).

* Mean ± SD for three measurements.

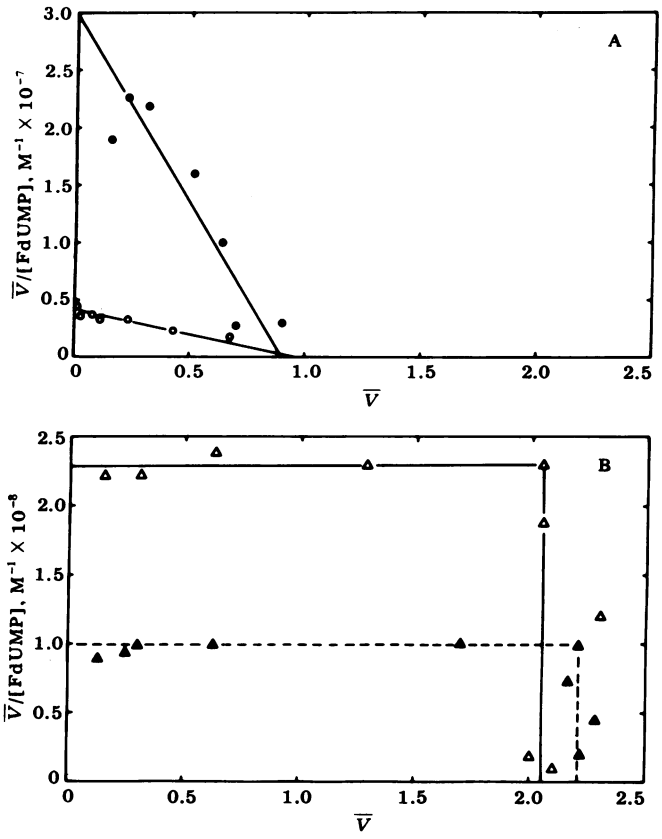

FIG. 2. Equilibrium dialysis of ternary complexes of thymidylate synthase. Thymidylate synthase in buffer A was dialyzed for 24 hr at 4°C in the presence of [^{3}H]FdUMP and one of the following: 40 μM MTX (○), 10 μM MTX(Glu$_1$) (●), 2 μM 7,8-H$_2$PteGlu$_5$ (△), or 10 μM 5,10-CH$_2$-H$_4$PteGlu$_5$ (▲). In *A* the enzyme concentration was 125 nM (specific activity 139 units/mg of protein) and the [^{3}H]FdUMP concentration varied from 30 nM to 1 μM. In *B*, the enzyme concentration was 14 nM (specific activity 55 units/mg of protein) and the [^{3}H]-FdUMP concentration varied from 3 to 400 nM.

thymidylate synthase (Fig. 2*B*). Moreover, with each folate species the binding of both moles of FdUMP was stoichiometric, with a K_d of less than 10 nM. Because of the very tight binding of FdUMP in the presence of the reduced pteroylpolyglutamates, as seen by the straight lines parallel to the abscissa in Fig. 2*B*, dissociation constants of less than 10 nM could not be further quantitated under these conditions.

DISCUSSION

Folates in mammalian cells exist mainly in the polyglutamyl form, predominately as the pentaglutamate (4, 6). Because the inactivation of thymidylate synthase by FdUMP *in vitro* has been shown to require the presence of a folate or antifolate cofactor (1), the effect of polyglutamates of MTX and folates on the binding of FdUMP to purified thymidylate synthase was examined. Equilibrium dialysis and enzyme inhibition studies revealed several differences among ternary complexes formed in the presence of MTX, MTX(Glu$_1$), and the reduced pteroylpolyglutamates H$_2$PteGlu$_5$ and 5,10-CH$_2$-H$_4$PteGlu$_5$. As determined by equilibrium dialysis, the binding of FdUMP to thymidylate synthase in the presence of MTX and MTX(Glu$_1$) was reversible, although considerably tighter with MTX(Glu$_1$) (K_d 27 nM) than with MTX (K_d 370 nM). Moreover, both MTX and MTX(Glu$_1$) stimulated FdUMP binding to only one site on the enzyme. Using *L. casei* thymidylate synthase and equilibrium dialysis in Tris buffer, Galivan *et al.* (34) have also demonstrated single-site reversible binding of FdUMP in the presence of MTX. In our study with H$_2$PteGlu$_5$ and 5,10-CH$_2$-H$_4$PteGlu$_5$, a second binding site for FdUMP was detected. Furthermore, with both polyglutamates, binding to each site was stoichiometric with a K_d of less than 10 nM. Similar results were reported by Galivan *et al.* for FdUMP binding in the presence of 5,10-CH$_2$-H$_4$PteGlu$_4$ (35), but with H$_2$PteGlu FdUMP bound stoichiometrically to only one enzyme site and less tightly to the second site (34).

Table 3 illustrated the effects of approximately physiological concentrations (low micromolar) of 5,10-CH$_2$-H$_4$PteGlu$_5$ and H$_2$PteGlu$_5$ on tight-binding inhibition of thymidylate synthase by FdUMP. The dihydro compound was of particular interest. On the basis of the data of Moran *et al.* (5) and assuming 0.68 ml of intracellular water per 10^9 cells (31), the total folate pool in L1210 cells can be calculated as approximately 8 μM. According to the results of Nixon *et al.* (32) and the mathematical model proposed by Jackson and Harrap (31), the concentration of dihydropteroylpolyglutamates in L1210 cells would increase by 2 orders of magnitude as a result of inhibition of dihydrofolate reductase, making them the predominant intracellular folates within 30 min after a 1 μM MTX exposure. Because H$_2$PteGlu was a competitive inhibitor of NADPH 5,10-CH$_2$-H$_4$PteGlu reductase with a K_i of 6 μM with respect to 5,10-CH$_2$-H$_4$PteGlu (36), the build-up of dihydropteroylpolyglutamates after MTX may have a self-reinforcing effect by inhibiting the flux of intracellular pteroylpolyglutamates into 5-methyltetrahydropteroyl polyglutamates. At the high intracellular concentrations generated by MTX treatment, dihydropteroylpolyglutamates could be important cofactors regulating the binding of FdUMP to thymidylate synthase. Consistent with this hypothesis were our findings that at low micromolar concentrations: (*i*) H$_2$PteGlu$_5$ reacted with FdUMP to produce 51% tight-binding inhibition of enzyme activity; and (*ii*) H$_2$PteGlu$_5$ stimulated the formation of very tight complexes to both FdUMP binding sites on thymidylate synthase as revealed by equilibrium dialysis. In addition, we previously reported that both H$_2$PteGlu$_5$ and 5,10-CH$_2$-H$_4$PteGlu$_5$, but not MTX(Glu$_1$), formed ternary complexes with FdUMP and thymidylate synthase that were stable to polyacrylamide gel electrophoresis under nondenaturing conditions (8). Galivan *et al.* (37) demonstrated that the complex formed with H$_2$PteGlu, FdUMP, and T2 bacteriophage-induced thymidylate synthase was partially stable to 6 M guanidine hydrochloride. However, the partially reduced state of H$_2$PteGlu$_5$ would be expected to preclude formation of a methylene bridge between the folate and the 5 position of FdUMP, thus preventing formation of a covalent ternary complex (2).

Binding of a limiting concentration of FdUMP in the presence of saturating concentrations of MTX(Glu$_1$) plus 5,10-CH$_2$-H$_4$PteGlu was approximately additive compared to binding in the presence of MTX(Glu$_1$) and 5,10-CH$_2$-H$_4$PteGlu alone (8). The FdUMP–MTX(Glu$_1$)–enzyme complex, although not inhibitory to the formation of the FdUMP–5,10-CH$_2$-H$_4$PteGlu–enzyme complex (8), also probably did not significantly contribute to the sequence-dependent synergism of the MTX–FUra combination. The intracellular accumulation of MTX polyglutamates and the distribution of MTX polyglutamyl chain lengths were not affected by the sequence of administration of MTX and FUra (data not shown).

A 1.4 log (25-fold) increase in the efficiency of the MTX–FUra combination in killing L1210 cells was observed when the cells were exposed to MTX prior to FUra, as compared to the opposite sequence (Table 1). It is possible that the intracellular levels of 5,10-CH$_2$-H$_4$PteGlu are insufficient for maximal FdUMP binding to thymidylate synthase in some cells. Indeed, Ullman *et al.* (38) have shown that the folate requirement for optimal growth of L1210 cells is less than that for maximal

cytotoxicity of 5-fluoro-2'-deoxyuridine. Our studies support the proposal that the enhanced cell kill seen with MTX pretreatment was mediated through MTX and MTX polyglutamate inhibition of dihydrofolate reductase, and consequently a trapping of intracellular folates as dihydropteroylpolyglutamates generated in the thymidylate synthase reaction. $H_2PteGlu_5$ induced the formation of very tight complexes with FdUMP and thymidylate synthase, which could counteract the potential effect of MTX in inhibiting FdUMP binding by lowering the intracellular levels of tetrahydrofolates. The less than additive effect on cell viability when cells were exposed to FUra prior to MTX (Table 1) may be related to both the build-up of dUMP (39, 40), and the decreased rate of depletion of the tetrahydrofolate pools resulting from inhibition of thymidylate synthase by FdUMP. The latter effect in maintaining the reduced folate pools would antagonize the inhibition of dihydrofolate reductase by MTX (41), because the function of this enzyme is to regenerate $H_4PteGlu_n$ from the $H_2PteGlu_n$ produced in the thymidylate synthase reaction. Unlike that seen with dihydropteroylpolyglutamates, a build-up and trapping of $5,10\text{-}CH_2\text{-}H_4PteGlu_n$ after inhibition of thymidylate synthase by FdUMP is probably prevented by utilization of the folate by 5,10-methylenetetrahydrofolate dehydrogenase (EC 1.5.1.5) (31) and other folate interconverting enzymes (42).

The MTX pretreatment of L1210 cells has also been noted to increase the incorporation of FUra into RNA, as well as to enhance its conversion to FdUMP (19). The relative role of an increased incorporation of FUra into RNA in relation to the sequence-dependent antitumor effects of the MTX–FUra combination is not clear, but one or both mechanisms may be acting in various cells to augment cell kill.

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