MOLECULAR PHARMACOLOGY, 21:224–230

MOL PHARM

Biochemical Interactions between *N*-(Phosphonacetyl)-L-aspartate and 5-Fluorouracil

CHI-MING LIANG, ROSS C. DONEHOWER, AND BRUCE A. CHABNER

Clinical Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Maryland 20205

Received July 20, 1981; Accepted September 22, 1981

SUMMARY

5-Fluorouracil (FU) blocks the synthesis of thymidylate by forming an active metabolite, 5-fluoro-2′-deoxyuridylate (FdUMP), which, in the presence of the cofactor 5,10-methylenetetrahydrofolate, binds to thymidylate synthetase (EC 2.1.1.45) to form a stable ternary complex. Exposure of logarithmically growing sarcoma 180 cells to FU (3–300 μM for 2 hr) caused a concentration-dependent decrease in cellular thymidylate synthetase available to bind [6-^{3}H]FdUMP (and to release tritium from [5-^{3}H]dUMP) in the assay solution. These results indicate that FU exposure caused the *de novo* formation of the ternary complexes and thus decreased the free thymidylate synthetase catalytic sites. When the cells were pretreated with *N*-(phosphonacetyl)-L-aspartate (PALA) (30–100 μM for 12 hr), exposure to the same concentration of FU (3–100 μM for 2 hr) led to a further decrease in free thymidylate synthetase catalytic sites, indicating the formation of increased inhibited ternary complexes. Increased complex formation could result from either increased 5-FdUMP formation or decreased formation of the competitive substrate dUMP. The level of dUMP in the PALA-pretreated cells (100 μM for 12 hr) was 68 ± 3 nmoles/10^9 cells, which was not much different from that in the control cells (79 ± 2 nmoles/10^9 cells). FU treatment (100 μM for 2 hr) increased the dUMP level more than 3-fold in the control cells but only 15% in the PALA-pretreated cells, indicating that PALA pretreatment prevented the accumulation of dUMP. After FU treatment (100 μM for 2 hr), PALA-pretreated cells contain 2.5-fold higher levels of 5-FdUMP than control cells. These results suggest that PALA, a transition-state analogue inhibitor of L-aspartate transcarbamylase, by blocking the synthesis of cellular pyrimidine nucleotides and increasing the formation of 5-FdUMP enhances FU inhibition of thymidylate synthetase.

INTRODUCTION

FU[1] has been widely used in the treatment of various solid tumors (1, 2). Its mechanism of action involves the formation of its nucleotides through the uracil metabolic pathways (1, 3–6). FdUMP is a potent inhibitor of thymidylate synthesis (7–9), whereas FUTP incorporated into RNA affects RNA maturation as well as function (10, 11). It is unclear which of these actions is most important in killing tumor cells. The inhibition of thymidylate synthetase (EC 2.1.1.45) by FdUMP results from the formation of a ternary complex including enzyme, FdUMP, and 5,10-CH_2-H_4folate (7, 9, 12, 13). FdUMP binding is competed for by the natural substrate dUMP and is slowly reversible (14).

Inhibition of thymidylate synthetase blocks the transformation of dUMP to thymidylate and increases the cellular level of dUMP (15, 16). This accumulation of dUMP may interfere with further binding of FdUMP (16). Drugs which block *de novo* synthesis of dUMP could potentially prevent dUMP accumulation and thus might enhance the effect of FdUMP on thymidylate synthetase. Among the possible inhibitors of *de novo* pyrimidine synthesis is PALA, a transition-state analogue inhibitor of L-aspartate transcarbamylase (17). By inhibiting L-aspartate transcarbamylase, one of the first enzymes in *de novo* pyrimidine biosynthesis, PALA decreases pyrimidine levels in a variety of mammalian cell lines in culture (18–20). In this paper, we first studied the effect of FU treatment on the thymidylate synthetase activity and on the cellular dUMP pool. The influence of PALA pretreatment on these biochemical effects of FU was then evaluated.

METHODS

Materials. FU was obtained from Calbiochem-Behring Corporation (La Jolla, Calif.). Dextran (average mol wt 282,000); activated charcoal, dUMP, 5-fluoro-2′-deoxyuridylate (FdUMP), and (±)-L-tetrahydrofolic acid were obtained from Sigma Chemical Company (St. Louis, Mo.). Bovine albumin Fraction V was purchased from

[1] The abbreviations used are: FU, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridylate; FUTP, 5-fluorouridine triphosphate; 5,10-CH_2-H_4folate, 5,10-methylene tetrahydrofolate; PALA, *N*-(phosphonacetyl)L-aspartate; PRPP, 5-phosphoribosyl-1-pyrophosphate.

0026-895X/82/010224-07$02.00/0
Copyright © 1982 by The American Society for Pharmacology and Experimental Therapeutics.
All rights of reproduction in any form reserved.

MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

MOL PHARM

MOLECULAR PHARMACOLOGY

Reheis Chemical Company (Phoenix, Ariz.); L-ascorbic acid was obtained from Fisher Scientific Company (Fair Lawn, N. J.), and PALA was obtained from the Drug Research and Development Division of the National Cancer Institute (Bethesda, Md.). PALA was dissolved in phosphate-buffered saline (0.067 M phosphate, 0.9% NaCl, without Ca^{2+}, pH 7.4) and titrated with 0.1 N NaOH solution until the acidity of the solution returned to pH 7.4. [6-^{3}H]FdUMP (22 Ci/mmole) and [5-^{3}H]dUMP (10 Ci/mmole) were purchased from Moravek Biochemicals (City of Industry, Calif.). [6-^{3}H]FdUMP was further purified by chromatography on DEAE-Sephadex using phosphate-buffered saline (pH 7.4) as eluents. The purity, as determined by high-performance liquid chromatography (Waters Associates, Inc., Milford, Mass.) on a C_{18} μBondapak column with 10 mM KH_2PO_4 (pH 4.5) as eluent, was greater than 90% for both [5-^{3}H]dUMP and the purified [6-^{3}H]FdUMP. Formaldehyde solution (37%) was obtained from J. T. Baker Chemical Company (Phillipsburg, N. J.). Bobby calf serum was obtained from Grand Island Biological Company (Grand Island, N. Y.), and Medium 199 (modified) was purchased from Flow Laboratories (McLean, Va.). Thymidylate synthetase from dichloromethotrexate-resistant *Lactobacillus casei* was obtained from New England Enzyme Center (Boston, Mass.); this enzyme preparation binds a maximum of 5.6 nmoles of FdUMP per milligram of protein.

The 5,10-CH_2-H_4folate solution was freshly prepared by addition of 50 μl of 1 M ascorbate (pH 6.5), 5 μl of 37% (v/v) formaldehyde, and 9.5 ml of a buffer solution [1 ml of 1 M phosphate (pH 7.2), 14 μl of 2-mercaptoethanol, 20 mg of bovine serum albumin, and 19 ml of water] to an vial containing 1.8 mg of *dl*-L-tetrahydrofolate. The mixture was freshly prepared daily and was kept under argon at 0° during the experiment. The albumin-coated charcoal suspension was prepared by mixing 10 g of acid washed-activated charcoal with 2.5 g of bovine serum albumin, 0.25 g of dextran, and 100 ml of ice-cold water. This suspension was diluted 1:5 before use.

Tissue culture. Sarcoma 180 cells were incubated with 10 ml of Medium 199 (modified) containing 10% Bobby calf serum in a tissue culture flask (Costar, Cambridge, Mass.). The cells were kept at 37° for 72–96 hr. The medium was changed every other day; cell number was determined on a Coulter counter. Under optimal growth conditions, the doubling time was 19 ± 2 hr. The cells were harvested by washing with phsophate-buffered saline (pH 7.4) followed by treatment with 3 ml of trypsin (0.05%) at 37° for 3 min; the proteolytic activity of trypsin was subsequently neutralized with 7 ml of cell culture medium. After centrifugation at 1000 × *g* for 3 min, the supernatant was discarded, and the cells were washed and kept at −20° for no more than 4 days. Immediately before enzyme activity measurement and binding assay were to be performed, the cell pellets were freeze-thawed three times and then suspended in 0.4 ml of phosphate-buffered saline (pH 7.4).

Thymidylate synthetase binding assay. The method used was a modification of that described by Moran *et al.* (16). Briefly stated, suitable amounts of cellular supernatants which contained thymidylate synthetase were mixed with 20 μl of 5,10-CH_2-H_4folate solution and 1.6 pmoles of [6-^{3}H]FdUMP (22 Ci/mmole) in a total volume of 100 μl. After 1 hr of incubation at 30°, 0.4 ml of ice-cold albumin-coated charcoal suspension was added, and the suspensions were mixed and centrifuged for 10 min at 1000 × *g*. A 0.1-ml portion of the supernatant was assayed for radioactivity in 8 ml of scintillation cocktail (Aquasol, New England Nuclear Corporation, Boston, Mass.).

Electrophoresis of macromolecule-bound [6-^{3}H]FdUMP complex. One hundred microliters of each supernatant obtained from the thymidylate synthetase binding assay were dried by blowing with nitrogen gas at 0°. The residue was suspended in 1 ml of solution containing 1% sodium dodecyl sulfate, 10% (v/v) glycerol, and 20 mM 2-mercaptoethanol. The suspensions were heated at 100° for 90 sec. Fifty microliters of the solution were electrophoresed on tube gels of 7.5% polyacrylamide as described by Weber and Osborn (21). The gels were sliced into 30 0.2-mm fractions, each of which was put into a paper cone, dried at 55° for 4 hr, and then examined for radioactivity by using a sample oxidizer (Packard Instrument Company, Downers Grove, Ill.). Recovery of radioactivity was greater than 90%.

Thymidylate synthetase activity assay. Standard or unknown quantities of thymidylate synthetase were added to a reaction mixture containing 1.6 pmoles of [5-^{3}H]dUMP (10 Ci/mmole) and 20 μl of freshly prepared 5,10-CH_2-H_4folate solution. When suitable amounts of thymidylate synthetase were present, the reaction was linear for 60 min. In this study, the samples were incubated for no more than 20 min at 37°. Reactions were terminated by adding 20 μl of a solution containing 1 volume of dUMP (3 mM) and 3 volumes of 2 N trichloroacetic acid. Two hundred microliters of an aqueous charcoal suspension (100 mg/ml) were subsequently added and the resulting suspension was centrifuged at 1000 × *g* for 10 min. A 50-μl aliquot of the supernatant was assayed for radioactivity in 8 ml of Aquasol. All assays were performed in duplicate.

Measurement of protein. Protein was determined according to the method of Lowry *et al.* (22) with bovine serum albumin as standard.

Isolation of free FdUMP from the cells. The FU-treated cells were suspended in 5 volumes of 1 M acetic acid and then freeze-thawed three times. After centrifugation at 1000 × *g* for 10 min, the cell pellets were further mixed with 2 volumes of 1 M acetic acid and heated in a boiling water bath for 90 sec. The supernatant fractions were combined, lyophilized, reconstituted with phosphate-buffered saline (pH 7.4), and adjusted to pH 7.4 with 1 N NaOH whenever necessary. The cellular dUMP and FdUMP were separated according to the method described by Moran *et al.* (16). Briefly stated, the supernatants were applied to a DEAE-cellulose column (0.7 × 6 cm) and then eluted with 30 ml of 0.1 M ammonium bicarbonate (pH 8.0); 3000 dpm of [5-^{3}H]dUMP were added as a marker. The fractions with radioactivity (dUMP) were collected, and FdUMP was then eluted with 10 ml of 0.3 M ammonium bicarbonate (pH 8.0). The elutes were lyophilized overnight and dissolved in 1 ml of phosphate-buffered saline (pH 7.4).

Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

FdUMP assay. The assay was based on competition between [6-^{3}H]FdUMP and unlabeled FdUMP for binding to *L. casei* thymidylate synthetase with subsequent removal of unbound [6-^{3}H]FdUMP by albumin-coated charcoal (16). Briefly stated 0.1 pmole of [6-^{3}H]FdUMP, 20 μl of 5,10-CH_2-H_4folate, and a suitable amount of *L. casei* thymidylate synthetase (capable of binding 0.05 pmole of [6-^{3}H]FdUMP) were added to a solution containing a standard or an unknown quantity of FdUMP in a total volume of 200 μl. After a 1-hr incubation at 30°, 1 ml of an ice-cold albumin-coated charcoal suspension was added and the suspension was mixed and centrifuged at 1000 × *g* for 10 min. A 0.8-ml portion of the supernatant was assayed for radioactivity in 15 ml of scintillation cocktail. The ratio of [6-^{3}H]FdUMP bound in the presence of a known quantity of unlabeled FdUMP to that in the absence of any unlabeled FdUMP was calculated (B/B_0). The logit of B/B_0 was subsequently plotted against the logarithm of unlabeled FdUMP concentration to generate of linear standard curve (Fig. 1A). This curve was used to calculate the quantity of FdUMP in cellular supernatant. The assay was capable of detecting a lower limit of 20 fmoles.

dUMP assay. L. casei thymidylate synthetase (capable of binding 0.025 pmole of [6-^{3}H]FdUMP) was added to a reaction mixture containing 2.5 pmoles of [5-^{3}H] dUMP (10 Ci/mole), 20 μl of 5,10-CH_2-H_4folate solution, and an unknown or standard concentration of dUMP in a total volume of 200 μl. The solution was mixed and incubated for 10 min at 37°. Reactions were terminated by adding 20 μl of a solution containing 1 volume of dUMP (3 mM) and 3 volumes of 2 N trichloroacetic acid. Two hundred microliters of an aqueous charcoal suspension (100 mg/ml) were subsequently added and the resulting suspensions was centrifuged at 1000 × *g* for 10 min. A 200-μl aliquot of the supernatant containing ^3H_2O liberated in the reaction was assayed for radioactivity in 8 ml of Aquasol. The ratio of the radioactivity (tritiated water) in the presence of unlabeled dUMP versus that in the absence of unlabeled dUMP (B/B_0) was obtained. The logit of B/B_0 was plotted against the logarithm of the concentration of unlabled dUMP to generate a linear standard curve (Fig. 1B). The curve was used to evaluate the unknown quantity of dUMP. The assay was capable of detecting a lower limit of 20 pmoles of dUMP.

RESULTS

Our initial efforts were directed at quantitating the concentration of FdUMP-binding sites in the cytosol of tumor cells folowing FU treatment. Incubation of Sarcoma 180 cell cytosol with 5,10-CH_2-H_4folate and [6-^{3}H] FdUMP produced a complex containing [6-^{3}H]FdUMP which could be separated from free [6-^{3}H]FdUMP by addition of albumin-coated charcoal, thus allowing quantitation of the FdUMP-binding sites in the cytosol. This complex has characteristics consistent with those of [6-^{3}H]FdUMP, thymidylate synthetase, and folate. The complex was stable to protein denaturation in 1% sodium dodecyl sulfate (100° for 2 min); it migrated to a position which correlated with a mol wt of 34,000 on sodium dodecyl sulfate polyacrylamide gel electrophoresis, a molecular weight equal to that of the subunit of thymidylate synthetase (Fig. 2). Mixing of unlabeled FdUMP (0.1 M) with the radioactive complex at 4° overnight caused little decrease in the radioactivity of this electrophoretic band, indicating that the complex was quite stable. When 5,10-CH_2-H_4folate was omitted from *in vitro* incubation, the complex formation decreased to less than 5%, suggesting that the complex was 5,10-CH_2-H_4folate-dependent (Fig. 2). Thus, the complex formed by 5,10-CH_2-H_4folate, [6-^{3}H]FdUMP, and the supernatant of Sarcoma 180 cells has the features previously identified with the thymidylate synthetase folate-FdUMP complex from purified enzyme of other sources (8, 13, 14, 23–25). The cytosol from Sarcoma 180 cells in exponential growth formed 2.8 ± 0.12 pmoles of complex per milligram of protein. Since

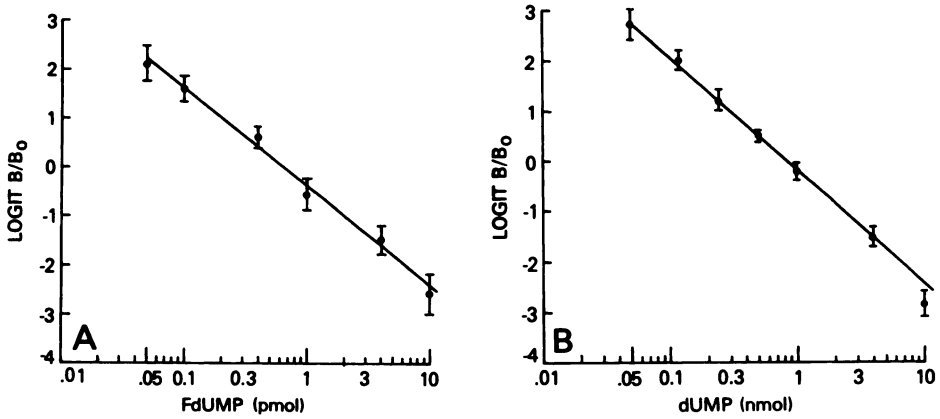

FIG. 1. *Relationship of concentration of unlabeled FdUMP and dUMP to logit B/B_0*

A. Standard curve relating concentration of unlabeled FdUMP to logit B/B_0. Each assay tube contained 0.1 pmole of [6-^{3}H]FdUMP, 5,10-CH_2-H_4folate, *Lactobacillus casei* thymidylate synthetase (bound 0.05 pmole of FdUMP), and varying amounts of unlabeled FdUMP as indicated. Details are provided under Methods. The points plotted are the mean values of three experiments. Vertical lines indicate standard error of the mean.

B. Standard curve relating concentration of unlabeled dUMP to logit B/B_0. Each assay tube contained 2.5 pmole of [5-^{3}H]dUMP, 5,10-CH_2-H_4folate, *L. casei* thymidylate synthetase (bound 0.025 pmole of FdUMP), and varying amounts of unlabeled dUMP as indicated. The assay was performed at 37° for 10 min. Details are provided under Methods. The points plotted are the mean values of three experiments. Vertical lines indicate standard error of the mean.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

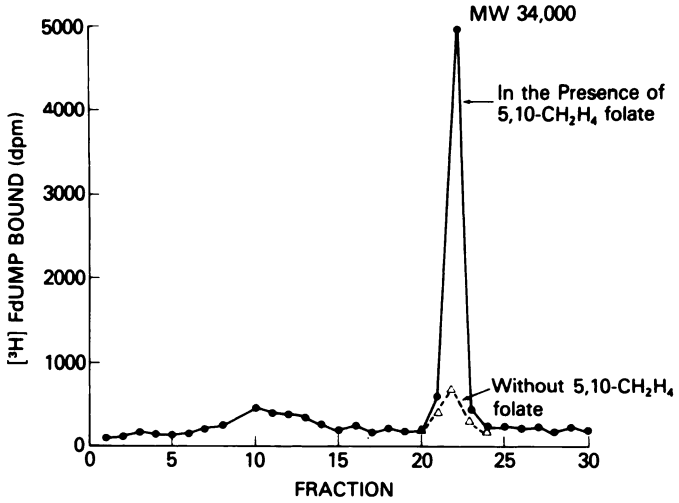

FIG. 2. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis of the [6-^{3}H]FdUMP-5,10-CH_2-H_4folate-thymidylate synthetase complex*

The complex was formed *in vitro* in the presence or absence of 5,10-CH_2-H_4folate as indicated. Details are provided under Methods.

the complex between thymidylate synthetase, 5,10-CH_2-H_4folate,and FdUMP contains a 1:2:2 stoichiometry (8, 13, 14, 24), this result suggests that there are at least as many free thymidylate synthetase-binding sites available to bind FdUMP.

In the cells treated with FU (3–300 μM), free thymidylate synthetase-binding sites, as measured by [6-^{3}H] FdUMP binding, were diminished (Fig. 3). This was not due to a cell-killing effect of FU, because neither cell number nor protein content was affected by the brief FU treatment (up to 100 μM for 2 hr). Since FU itself has little effect on synthesis of thymidylate synthetase (1, 12) and is known to form FdUMP in cells, the decrease in number of binding sites for [6-^{3}H]FdUMP is most likely due to the occupation of these sites by FdUMP following FU treatment. This decrease of binding sites was dependent on the doses of FU (3–300 μM for 2 hr). A maximum of 80% of the binding sites (presumed to represent thymidylate synthetase) were occupied when the cells were treated with 100 μM of FU for 2 hr (Fig. 4).

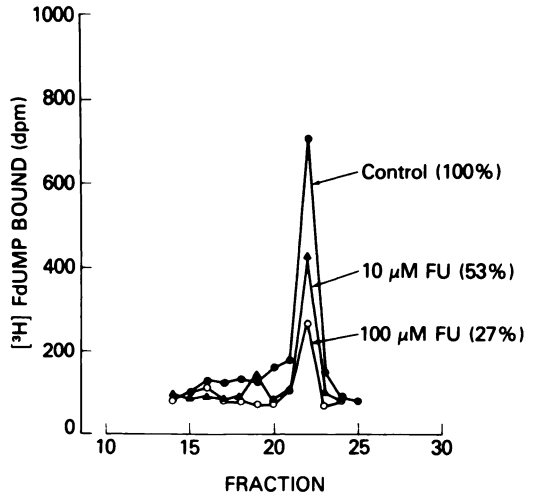

FIG. 3. *Effect of FU treatment on thymidylate synthetase binding sites*

The radioactive complex was formed *in vitro* by incubating [6-^{3}H] FdUMP, 5,10-CH_2-H_4folate, and the cytosol of cells (with and without FU pretreatment) as described under Methods. The amounts of FU used are as indicated.

The catalytic activity of thymidylate synthetase in cell cytosol decreased in direct proportion to the decrease in [6-^{3}H]FdUMP binding sites. The cytosol of untreated Sarcoma 180 cells catalyzed 1.2 pmoles of dUMP/min per milligram of protein *in vitro*. In the cells treated with FU, the activity of the enzyme decreased in a dose-dependent manner. This decrease of thymidylate synthetase activity correlated well with the decrease in thymidylate synthetase-binding sites (Fig. 5).

The cytosol from PALA (100 μM for 12–18 hr)-pretreated cells formed 2.76 ± 0.15 pmoles of complex per milligram of protein, which was insignificantly different from that in the control cells (2.82 ± 0.12 pmoles/mg of protein). Incubation of cells with PALA (100 μM for 12–18 hr), an inhibitor of *de novo* pyrimidine synthesis, therefore did not affect the number of thymidylate synthetase-binding sites (and catalytic activity of the enzyme). However, the PALA pretreatment augmented the inhibition of thymidylate synthetase. Thirty micromolar FU (2 hr) caused a 50% occupation of [6-^{3}H]FuUMP binding sites in the control cells, whereas 3 μM FU achieved the same percentage decrease in PALA (100 μM for 12 hr)-pretreated cells (Fig. 4). FU also caused a greater inhibition of thymidylate synthetase activity in the PALA-pretreated cells than in the control cells (Fig. 5).

This potentiating effect of PALA did not occur immediately. Incubation of cells with PALA for less than 6 hr showed little, if any, synergistic effect with FU. Only after exposure of the cells to PALA for 12 hr (or longer) was the synergistic effect of PALA and FU observed.

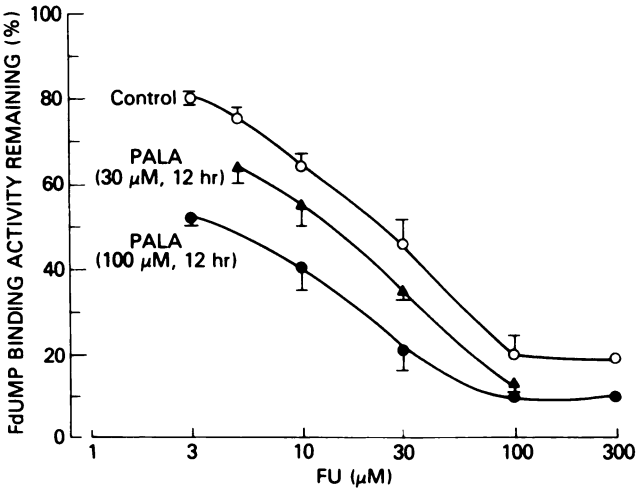

FIG. 4. *Effect of PALA on FU-induced inhibition of thymidylate synthetase binding activity*

Cells were pretreated with PALA for 12 hr and then incubated with FU for 2 hr. The amounts of FU and PALA used are as indicated. The binding activity of thymidylate synthetase was assayed by incubating cellular cytosol with [6-^{3}H]FdUMP and 5,10-CH_2-H_4folate *in vitro*. Details are provided under Methods. The points plotted are the mean values of at least three experiments. Vertical lines indicate standard error of the mean.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

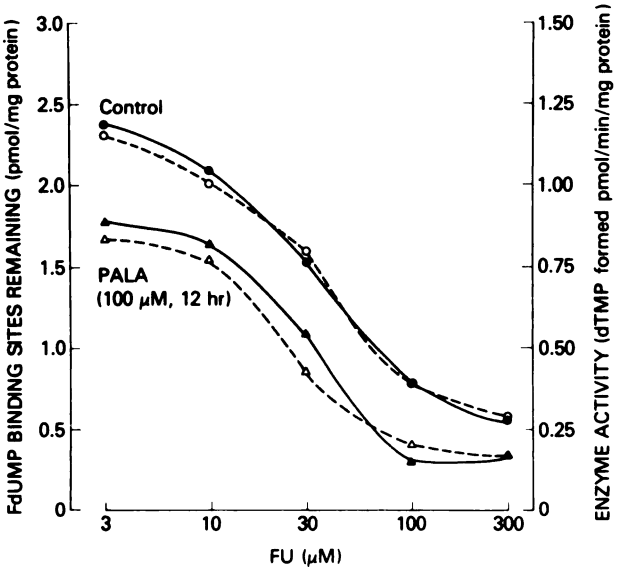

FIG. 5. *Effect of PALA on FU-induced inhibition of thymidylate synthetase (both binding and catalytic activity)*

The cells were pretreated with PALA for 12 hr and then incubated with FU for 2 hr. The amounts of FU and PALA used are as indicated. the cellular cytosol was partly assayed for binding activity and partly assayed for catalytic activity. The catalytic activity (——) was assayed by incubating cellular cytosol with [5-^{3}H]dUMP, and 5,10-CH_2-H_4folate at 37° for 10 min. The binding activity (- - -) was assayed by incubating cellular cytosol with [6-^{3}H]FdUMP and 5,10-CH_2-H_4folate at 30° for 1 hr. The points plotted are the mean values of two duplicate detemina-tions.

This delay in the effect of PALA was not due to the duration which PALA required to inhibit L-aspartate transcarbamylase because, even in the cells treated with PALA for 2 hr, more than 90% of the aspartate transcarbamylase activity had already been inhibited. In view of the over-all effect of PALA on pyrimidine biosynthesis, the delayed effect of PALA may be due to the time required to delete dUMP and/or enhance FdUMP formation in the cells.

Free cellular dUMP was measured by an assay method based on the competition between unlabeled dUMP and [5-^{3}H]dUMP for the catalytic sites of *L. casei* thymidylate synthetase (Fig. 1B). This method gave a sensitivity of 20 pmoles, which is comparable to that described by Moran *et al.* (16) but with the advantage that the new method is less time-consuming. Sarcoma 180 cells contained 79 ± 2 nmoles of dUMP per 10^9 cells. Incubation of the cells with FU (100 μM for 2 hr) caused a 3-fold increase of dUMP. This increase in dUMP levels (after FU treatment) is consistent with the increase reported by others (15, 16). In the cells pretreated with PALA (100 μM for 12 hr), the level of dUMP, 68 ± 3 nmoles/10^9 cells, was not significantly different from that in the control cells (79 ± 2 nmoles). However, PALA pretreatment prevented an increase in dUMP after FU, since dUMP levels rose only 15% in cells exposed to PALA and then FU (Table 1).

PALA could also increase FdUMP formation in cells by reducing the intracellular production of pyrimidines and thus reducing competitive substrates in the FU activation pathway.

Incubation of the cells with FU alone (100 μM for 2 hr) resulted in a free FdUMP level of 60 pmoles/10^9 cells. However, in the PALA (100 μM for 12 hr)-pretreated cells the same concentration of FU resulted in a concentration of FdUMP (148 pmoles/10^9 cells) which was 2.5 times higher than that in cells treated with FU alone. Thus, the formation of FdUMP:thymidylate synthetase complex was enhanced both by a rise in FdUMP concentration and a blunted increase in the competitive substrate dUMP (Table 1).

DISCUSSION

FdUMP, an active inhibitory metabolite of the antitumor drug FU, reacts with 5,10-CH_2-H_4folate and thymidylate synthetase to form a ternary-inhibited complex (7–9, 13). The formation of the complex prevents transformation of dUMP to dTMP and is considered to be one of the important actions of FU (1). A direct approach to assaying the effect of FU treatment on thymidylate synthetase involves treatment of the cells with [6-^{3}H]FU and then quantitative isolation of the radioactive ternary complex from the cytosol. However, we found that this process was cumbersome and required considerable amounts of [6-^{3}H]FU. In addition, the [6-^{3}H]FU was also incorporated into other radioactive macromolecules (e.g., RNA) which made the purification and quantitative measurement of the complexes difficult. As an alternative,we measured the effect of FU treatment on thymidylate synthetase catalytic sites by treating the cells with unlabeled FU and then titrating the remaining free binding sites with [6-^{3}H]FdUMP *in vitro* (Fig. 3). The radioactive complex was easily separated from free [6-^{3}H]FdUMP by albumin-coated charcoal. This method is simple, direct, sensitive to the lower limit of binding sites, and gives reproducible results. Figure 4 shows that FU treatment causes a dose-dependent decrease of thymidylate synthetase available to bind [6-^{3}H]FdUMP, indicating that the enzyme-binding sites are preoccupied by FdUMP following FU treatment. The decrease of thymidylate synthetase binding sites correlated with diminished thymidylate synthetase activity (Fig. 5), suggesting that the catalytic sites are closely related to, if not identical with, the [6-^{3}H]FdUMP-binding sites.

Myers *et al.* (15) reported that there was a progressive increase in P1534 tumor cell dUMP levels following FU administration. Moran and co-workers (16) have also

TABLE 1

FdUMP and dUMP Concentrations in Sarcoma 180 Cells

	Control	FU (100 μM, 2 hr)	PALA (100 μM, 12 hr)	PALA + FU
dUMP[a] (nmoles/10^9 cells)	79 ± 2	241 ± 5	68 ± 3	81 ± 3
FdUMP[a] (pmoles/10^9 cells)	—	60 ± 4	—	148 ± 10

[a] Values are means ± standard deviation of three to five experiments.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

found the same phenomenon in CCRF-CEM cell lines. Our observation that FU treatment increased dUMP levels in Sarcoma 180 cells by several-fold (Table 1), therefore, is consistent with these reports. This accumulation of dUMP may reach a level sufficient to retard the inhibition of thymidylate synthetase by FdUMP (16). Indeed, Myers *et al.* (15) found that the resumption of DNA synthesis following FU treatment coincided with the peak of dUMP levels.

PALA, a transition-state analogue inhibitor of aspartate transcarbamylase, blocks the biosynthesis of pyrimidines and decreases UTP levels *in vitro* (20). Exogenous uridine rapidly reverses the cytotoxicity of PALA, thus indicating that pyrimidine deprivation is the primary cytotoxic effect of PALA (26). Recently, it has been found that pretreatment of the cells with PALA increased the antitumor activity of FU and increases FU incorporation into RNA (27, 28). However, whether this enhancement of FU effect by PALA is due to the inhibition of pyrimidines biosynthesis is not yet established. In this study, we found that inhibition of thymidylate synthetase (both binding sites and catalytic sites) following FU treatment was greater in the cells pretreated with PALA than in the control cells (Fig. 4). Since PALA alone did not affect the thymidylate synthetase activity in control experiments, these results indicate that PALA potentiates the effect of FU treatment on thymidylate synthetase. Analysis of the dUMP level showed that the basal dUMP in PALA-pretreated cells was only 15% less than that in the control cells. However, when both PALA-pretreated cells and control cells were treated with FU, FU caused a 3-fold increase of dUMP in control cells but only a 15% increase in PALA-pretreated cells (Table 1). Since dUMP competes with FdUMP for the thymidylate synthetase binding sites, it is likely that the prevention of dUMP accumulation by PALA accounts partly, if not solely, for the synergistic effect of PALA and FU on occupancy of FdUMP-binding sites.

The influence of PALA on the FdUMP level has not been reported previously. We found that the acetic acid extract of PALA-pretreated cells contains 2.5 times more FdUMP than that of the cells without PALA treatment. FU is converted to its nucleotides through the same pathways which metabolize UMP to UTP and to deoxyribonucleotides (1, 3–6). Drugs which decrease the intracellular concentration of these competitor pyrimidine precursors may allow increased formation of FU deoxyribonucleotides. In addition, a second mechanism could be invoked for increased FdUMP formation following PALA. Recently, Cadman *et al.* (29) found that methotrexate, which increased PRPP, a phosphoribosyl donor for uracil, also increased the formation of intracellular FU nucleotides. Furthermore, this increase of FU nucleotides in cells exposed to methotrexate was diminished by hypoxanthine, a PRPP acceptor. By blocking L-aspartate transcarbamylase, PALA may cause an accumulation of PRPP (30) and thus affect FU metabolism.

In recent years, much interest has centered on combination chemotherapy for the treatment of neoplasms. With few exceptions, clinical protocols for multiple-drug cancer chemotherapy have been designed empirically. The combination of FU and PALA has an attractive biochemical rationale and is supported by experimental evidence of a favorable biochemical interaction. In the present study, PALA, an L-aspartate transcarbamylase inhibitor, prevented the accumulation of dUMP and enhanced the formation of FdUMP; the cumulative effect was potentiation of the formation of the enzyme:FdUMP:folate complex. This potentiating effect required PALA pretreatment for a period of 12–18 hr, suggesting that the specific time sequence of giving PALA and FU may be an important factor in combination chemotherapy with these two drugs.

PALA may also enhance the formation of FUTP from FU. Martin *et al.* (27) and Kufe (28) have observed that PALA pretreatment increases the incorporation of FU into RNA, thus potentiating the second arm of the dual effects of FU on nucleic acids. It is important to determine whether the experimental synergism of these and other combinations is translated into potentiated antitumor activity in experimental and clinical trials.

ACKNOWLEDGMENTS

We are grateful to Dr. Stanley E. Shackney and Ms. Sandy Occhipinti for providing the Sarcoma 180 cells. We would like to thank Dr. Charles Erlichman for assaying the activity of L-aspartate transcarbamylase, Dr. Yung-Pin Liu for helpful discussion and advice, and Ms. Kathy Moore for typing the manuscript.

REFERENCES

1. Heidelberger, C. *Antineoplastic and Immunosupressive Agents*, Part II. Springer-Verlag, Berlin, 193–231 (1975).
2. Heidelberger, C. *Cancer Medicine.* Lea & Febiger, Philadelphia, 768–791 (1973).
3. Bertani, L. E., A. Haggmark, and P. Reichard. Enzymatic synthesis of deoxyribonucleotides. II. Formation and interconversion of deoxyuridine phosphates. *J. Biol. Chem.* **238**:3407–3413 (1963).
4. Kent, R. J., and C. Heidelberger. Fluorinated pyrimidines. XL. The reduction of 5-fluoridine 5′-diphosphate by ribonucleotide reductase. *Mol. Pharmacol.* **8**:465–475 (1972).
5. Labow, R., and F. Maley. The conversion of deoxyuridine 5′-monophosphate to deoxyuridine 5′-triphosphate in normal and regenerating rat liver. *Biochem. Biophys. Res. Commun.* **29**:136–142 (1967).
6. Reyes, P. The synthesis of 5-fluoruridine 5′-phosphate by a pyrimidine phosphoribosyltransferase of mammalian origin. I. Some properties of the enzyme from P1534J mouse leukemic cells. *Biochemistry* **8**:2057–2062 (1969).
7. Danenberg, P. V., R. J. Langenbach, and C. Heidelberger. Structures of reversible and irreversible complexes of thymidylate synthetase and fluorinated pyrimidine nucleotides. *Biochemistry* **13**:926–933 (1974).
8. Santi, D. V., and C. S. McHenry. 5-Fluoro-2′-deoxyuridylate: covalent complex with thymidylate synthetase. *Proc. Natl. Acad. Sci. U. S. A.* **69**:1855–1857 (1972).
9. Santi, D. V., C. S. McHenry, and H. Sommer. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry* **13**:471–481 (1974).
10. Wilkinson, D. S., and H. C. Pitot. Inhibition of ribosomal ribonucleic acid maturation in Novikoff hepatoma cells by 5-fluorouracil and 5-fluorouridine. *J. Biol. Chem.* **248**:63–68 (1973).
11. Wilkinson, D. S., T. D. Tisty, and R. J. Hanas. The inhibition of ribosomal RNA synthesis and maturation in Novikoff hepatoma cells by 5-fluorouridine. *Cancer Res.* **35**:3014–3020 (1975).
12. Cohen, S. S., J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein. The mode of action of 5-fluorouracil and its derivatives. *Proc. Natl. Acad. Sci. U. S. A.* **44**:1004–1012 (1958).
13. Aull, J. L., J. A. Lyon, and R. B. Dunlap. Separation, identification, and stoichiometry of the ternary complexes of thymidylate synthetase. *Arch. Biochem. Biophys.* **165**:805–808 (1974).
14. Danenberg, P. V., and K. D. Danenberg. Effect of 5,10-methylenetetrahydrofolate on the dissociation of 5-fluoro-2′-deoxyuridylate from thymidylate synthetase: evidence for an ordered mechanism. *Biochemistry* **17**:4018–4024 (1978).
15. Myers, C. E., R. C. Young, and B. A. Chabner. Assay of 5-fluorodeoxyuridine 5′-monophosphate and deoxyuridine 5′-monophosphate pools following 5-fluorouracil. *Cancer Res.* **34**:2682–2688 (1974).
16. Moran, R. G., C. P. Spears, and C. Heidelberger. Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate, and thymidylate synthetase. *Proc. Natl. Acad. Sci. U. S. A.* **76**:1456–1460 (1979).
17. Collins, K. D., and G. R. Stark. Aspartate transcarbamylase. Interaction with

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012

the transition state analogue *N*-(phosphonacetyl)-L-aspartate. *J. Biol. Chem.* **246**:6599–6605 (1971).
18. Swyryd, E. A., S. S. Seaver, and G. R. Stark. *N*-(phosphonacetyl)-L-aspartate, a potent transition state analog inhibitor of aspartate transcarbamylase, blocks proliferation of mammalian cells in culture. *J. Biol. Chem.* **249**:6945–6950 (1974).
19. Yoshida, T., G. R. Stark, and N. J. Hoggenraad. Inhibition by *N*-(phosphonacetyl)-L-aspartate of aspartate transcarbamylase activity and drug-induced cell proliferation in mice. *J. Biol. Chem.* **249**:6951–6955 (1974).
20. Johnson, R. K., E. A. Swyryd, and G. R. Stark. Effects of *N*-(phosphonacetyl)-L-aspartate on murine tumors and normal tissues *in vivo* and *in vitro* and the relationship of sensitivity to rate of proliferation and level of aspartate transcarbamylase. *Cancer Res.* **38**:371–378 (1978).
21. Weber, K., and M. Osborn. The reliability of molecular weight determinations by dodecyl sulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244**: 4406–4412 (1969).
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
23. Dunlap, R. B., N. G. L. Harding, and F. M. Huennekens. Thymidylate synthetase from amethopterin-resistant *Lactobacillus casei*. *Biochemistry* **10**:88–97 (1971).
24. Galivan, J. H., G. F. Maley, and F. Maley. Factors affecting substrate binding in *Lactobacillus casei* thymidylate synthetase as studied by equilibrium dialysis. *Biochemistry* **15**:356–362 (1976).
25. Lockshin, A. and P. V. Danenberg. Hydrodynamic behavior of human and bacterial thymidylate synthetases and thymidylate synthetase-5-fluoro-2′-deoxyuridylate-5,10-methylenetetrahydrofolate complexes: evidence for large conformational changes during catalysis. *Biochemistry* **19**:4244–4251 (1980).
26. Johnson, R. K. Reversal of toxicity and antitumor activity of *N*-(phosphonacetyl)-L-aspartate by uridine or carbamyl-DL-asparate *in vivo*. *Biochem. Pharmacol.* **26**:81–84 (1977).
27. Martin, D. S., R. Nayak, R. C. Sawyer, R. L. Stolfi, C. W. Young, T. Woodcock, and S. Spiegelman. Enhancement of 5-fluororuacil chemotherapy with emphasis on the use of excess thymidine. *Cancer Bull.* **30**:219–224 (1979).
28. Kufe, D. Metabolic enhancement of 5-fluorouracil incorporation into human tumor cell RNA (abstr.). *Proc. Am. Assoc. Cancer Res.*, 265 (1980).
29. Cadman, E., R. Heimer, and C. Benz. The influence of methotrexate pretreatment on 5-fluorouracil metabolism in L1210 cells. *J. Biol. Chem.* **256**:1695–1704 (1981).
30. Santi, D. V. Perspectives on the design and biochemical pharmacology of inhibitors of thymidylate synthetase. *J. Med. Chem.* **23**:103–111 (1980).

Send reprint requests to: Dr. Chi-Ming Liang, Clinical Pharmacology Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. 20205.

MOL PHARM
MOLECULAR PHARMACOLOGY
aspet
Downloaded from molpharm.aspetjournals.org at Universidade do Estado do Rio de Janeiro on December 6, 2012