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Thymidylate Synthetase Levels as a Factor in 5-Fluorodeoxyuridine and Methotrexate Cytotoxicity in Gastrointestinal Tumor Cells

WENDY L. WASHTIEN

Department of Pharmacology and the Cancer Center, Northwestern University Medical School, Chicago, Illinois 60611

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SUMMARY

Thymidylate synthetase levels in five human gastrointestinal tumor cell lines (two colon, two colorectal, one stomach) were determined. Titration of the enzyme in cell cytosol using the active-site titrant, 5-fluoro-2′-deoxyuridine-5′-monophosphate, demonstrated a 20-fold variation in the level of this enzyme among the tumor lines. Titrations performed in the presence or absence of added methylenetetrahydrofolate gave the same values for enzyme content. The cytotoxicity of 5-fluorodeoxyuridine to these cell lines (expressed as EC_{50} values) varied from 0.44 nM for SW 403 cells to 16 nM for HuTu 80 cells, and in all cases was reversed by the addition of thymidine. The concentration of 5-fluorodeoxyuridine required for cytotoxicity correlated directly ($r = 0.98$) with the level of thymidylate synthetase in the particular cell line. An inverse correlation ($r = -0.95$) was observed between the concentration of methotrexate producing cytotoxicity in these cell lines and their thymidylate synthetase levels. The cells were found to contain similar levels of dihydrofolate reductase and to possess normal transport capability for methotrexate.

INTRODUCTION

A growing concern in cancer chemotherapy is the apparent heterogeneity among tumors and the extent to which this variability may compromise the efficacy *in vivo* of a chemotherapeutic agent of proven value *in vitro*. One area in which tumors have been shown to vary is in their enzyme content; much work on hepatoma cell lines, and more recently on colon tumors, indicates that the malignant state is accompanied by dramatic changes in many enzyme activities (1, 2). Equally important, resistance to chemotherapeutic agents has often been linked to changes in enzyme activities which either are required for drug activation or represent the ultimate target of the drug (3).

The enzyme dTMP synthetase[1] (EC 2.1.1.45) catalyzes Reaction *2* shown in Fig. 1. This enzymatic reaction represents the sole *de novo* pathway for thymidine formation in the cell, involving the conversion of deoxyuridylate to thymidylate with concomitant oxidation of the CH_2FH_4 cofactor to dihydrofolate. Tetrahydrofolate is then regenerated in a subsequent reaction.

Because of the pivotal role of dTMP synthetase in DNA synthesis, many chemotherapeutic agents have been designed to inhibit this enzyme. The fluorinated nucleotide FdUMP, which is formed from FdUrd by the action of thymidine kinase, directly inhibits dTMP synthetase through the formation of a ternary complex between FdUMP, CH_2FH_4, and the enzyme (4). Fluorouracil, after intracellular conversion to FdUMP, is also thought to act in this fashion, although alternative mechanisms for its cytotoxicity have been suggested (5). The direct interaction between FdUMP, CH_2FH_4, and dTMP synthetase required for cytotoxicity suggests that the level of enzyme (as well as the levels of FdUMP and CH_2FH_4) can play a critical role in determining drug efficacy. Some cell lines which show resistance to FdUrd have been found to contain increased levels of dTMP synthetase (6), and we have previously demonstrated a difference in dTMP synthetase level between S49 lymphoma cells and HTC cells which was reflected by a difference in the cytotoxicity of FdUrd to these cells (7).

The level of dTMP synthetase activity is also important to the cytotoxicity of another chemotherapeutic agent, MTX, which does not interact directly with dTMP synthetase. Rather, MTX binds stoichiometrically to dihydrofolate reductase (EC 1.5.1.3), the enzyme responsible for regeneration of tetrahydrofolate from dihydrofolate (Fig. 1, Reaction *3*). Cytotoxicity is thought to result from depletion of cellular tetrahydrofolate, which is required for initial steps in purine biosynthesis as well as for continued thymidylate formation. Although the inability to regenerate tetrahydrofolate is a direct result of the MTX-dihydrofolate reductase interaction, the actual depletion of this compound in the cell is a result of the dTMP synthetase reaction. Bacterial mutants, defi-

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[1] The abbreviations used are: dTMP synthetase, thymidylate synthetase; CH_2FH_4, 5,10-methylenetetrahydrofolate; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; FdUrd, 5-fluoro-2′-deoxyuridine; MTX, methotrexate.

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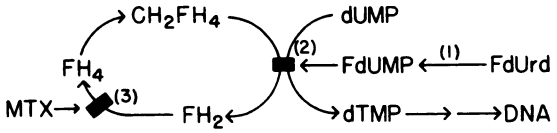

FIG. 1. *Sites of action of FdUrd and MTX*

Enzymes involved are (*1*) thymidine kinase, (*2*) thymidylate synthetase, and (*3*) dihydrofolate reductase. The abbreviations used are: FH_2, 7,8-dihydrofolate; FH_4, 5,6,7,8-tetrahydrofolate.

cient in dTMP synthetase but grown in media supplemented with thymidine, can grow in the presence of MTX (8). More recently, mutant mouse mammary tumor cells containing reduced dTMP synthetase activity were shown to exhibit increased resistance to MTX (9). Several other studies in which dTMP synthetase levels were artificially altered have also suggested a relationship between this enzyme activity and MTX sensitivity (10, 11).

In this study, the variability in dTMP synthetase levels in several independently derived gastrointestinal tumor cell lines was examined. The effect of such variability of FdUrd cytotoxicity, which involves direct interaction with the enzyme, and on MTX cytotoxicity, which depends indirectly on the reaction catalyzed by dTMP synthetase, was investigated.

MATERIALS AND METHODS

Chemicals. [6-^{3}H]FdUrd and [6-^{3}H]FdUMP (specific activity, 18 Ci/mmole) were purchased from Moravek Biochemicals (City of Industry, Calif.). Tetrahydrofolic acid was prepared from folic acid (Sigma Chemical Company, St. Louis, Mo.) by the procedure of Hatefi *et al.* (12). Solutions of CH_2FH_4 (pH 9.5) were stored under vacuum at −80° in 0.12 M β-mercaptoethanol and 30 mM formaldehyde. The actual concentration of CH_2FH_4 was determined spectrophotometrically (13). [3′,5′,7(N)^{3}H] MTX (specific activity, 14 Ci/mmole), obtained from Moravek Biochemicals, and nonlabeled MTX, obtained from Sigma Chemical Company, were purified before use by DEAE-column chromatography (14). All other chemicals were purchased from Sigma Chemical Company unless otherwise specified.

Cells and media. Human carcinoma cell lines were obtained from the following sources: HuTu 80 (15) and HT 29 (16), Naval Biosciences Laboratory (Berkeley, Calif.)[2]; WIDR (17), Lederle Laboratories (Pearl River, N. Y.); SW 403 and SW 480 (18), Scott and White Clinic (Temple, Tex.). Initial characterization of these cell lines has been described in the indicated references.

All cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (KC Biologicals, Kansas City, Mo.) at 37° in an atmosphere of 92% O_2–8% CO_2. The doubling times for the various cell lines under these conditions were as follows: HuTu 80 (21 hr); HT 29 (28 hr); WIDR (27 h); SW 480 (25 hr); SW 403 (28 hr for the first 50 hr of growth, then slowing to 60 hr). Cells were monitored semimonthly for the absence of mycoplasma using the Hoescht staining method (19). Cell densities were measured with a Model ZBI Coulter counter (Coulter Electronics, Inc., Hialeah, Florida).

Cytotoxity (EC_{50}) determinations. Determinations of the growth of tumor cells in the presence of drugs were conducted in CoStar (Cambridge, Mass.) 24-well plates. Experiments were begun by the addition of 1 ml of medium containing 0.7–1 × 10^5 monodispersed growing cells to wells in which 0–50 μl of the particular drug had been placed. The cells were incubated at 37° in a humidified 8% CO_2 atmosphere for 72–96 hr. SW 403 cells, because of their slow growth rate, were incubated for 168 hr. The monolayers of cells were then removed by trypsinization and counted in suspension. Cell number was determined on a Coulter counter and maximally increased 10- to 15-fold over that inoculated. EC_{50} values refer to that concentration of inhibitor necessary to inhibit cell growth by 50% as compared with control cells grown under identical conditions except that the inhibitor was omitted.

Measurement of dTMP synthetase binding sites. Cells (2–3 × 10^5) were seeded in 25 cm^2 tissue culture flasks, and allowed to attach and enter exponential growth (final density 1–3 × 10^5 cells/cm^2). Cells were removed from the flask by gentle trypsinization, washed twice with cold phosphate-buffered saline (0.137 M NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.77 mM KH_2PO_4, 0.68 mM $CaCl_2$, and 0.48 mM $MgCl_2$) (20), and resuspended in ice-cold 20 mM sodium phosphate (pH 7.3) containing 10 mM β-mercaptoethanol and 1.6 M formaldehyde. The concentration of resuspended cells was within the range of 1.5–3 × 10^6 cells/ml. The cell suspension was sonicated (Branson sonifier, Setting 4) and cell debris was removed by centrifugation (4°, 1000 × g for 5 min). Following the addition of [6-^{3}H]FdUMP (18 Ci/mmole, final concentration 0.11 μM) to the cell sonicate the mixture was incubated at 25° for 1 hr to allow formation of the dTMP synthetase:FdUMP:CH_2FH_4 complex. Incubations were performed without the addition of exogenous CH_2FH_4 unless indicated.

Macromolecule-bound radioactivity present after this incubation was measured by trichloroacetic acid precipitation onto glass-fiber filters as previously described (7) and represents the number of active sites of dTMP synthetase. This value can be used as a measure of dTMP synthetase levels (21). Radioactivity measurements were performed in 20-ml glass vials using 3a70b counting fluid (Research Products International, Elk Grove Village, Ill.) and a Beckman LS9000 liquid scintillation counter.

Measurements of [^{3}H]FdUMP binding to thymidylate synthetase in cells exposed to [6−^{3}H]FdUrd were performed as previously described (7).

Dihydrofolate reductase activity. Cells were harvested and crude enzyme extracts were prepared for assay of dihydrofolate reductase activity as described by Chello *et al.* (22). Enzyme activity at 25° was determined by measuring the combined decrease in absorbance at 340 nm from the conversion of NADPH and dihydrofolate to NADP and tetrahydrofolate, respectively (23). Protein concentrations were determined by the method of Lowry *et al.* (24).

[2] These cells were produced with support from the National Cancer Institute, Biological Carcinogenesis Branch, Division of Cancer Cause and Prevention, under the auspices of the Office of Naval Research and the Regents of the University of California.

[^{3}H]MTX transport. Cells were seeded at 2×10^5 cells/60-mm tissue culture dish 3–4 days prior to the day of the experiment. One hour before the beginning of an experiment, the complete medium was replaced with medium without serum. The time course of uptake of [^{3}H]MTX was measured as described by Schilsky *et al.* (25), using an extracellular drug concentration of 1 μM.

Drug efflux was measured as described by Sirotnak (26). Cells were loaded with [^{3}H]MTX by incubation with the drug (1 μM) for 1 hr. The cells were then cooled to 0° and washed twice with ice-cold phosphate-buffered saline. After addition of medium with serum, the cells were incubated at 37° in an 8% CO_2 atmosphere, and the amount of intracellular [^{3}H]MTX remaining was assayed as above. The [^{3}H]MTX remaining after 1 hr was taken to represent non-exchangable drug which was bound to dihydrofolate reductase (26).

RESULTS

dTMP synthetase levels in human gastrointestinal tumor cells. The level of dTMP synthetase in five independently derived human gastrointestinal tumor cell lines was determined by titration of the enzyme in cell sonicates, using [^{3}H]FdUMP, a specific active-site probe for this enzyme (27). The number of FdUMP binding sites can be taken as a direct measure of the enzyme level in the cells. The results, shown in Table 1, demonstrate a wide variation in the amount of enzyme present in the various cell lines. As indicated, this variability does not merely reflect a difference in the absolute amount of protein per cell, since it is not lost when the data are expressed as binding sites per microgram of protein. Although the SW 403 cell line, which contains the least amount of dTMP synthetase, is the most slowly growing of the five cell lines (see Materials and Methods), there is no over-all correlation between enzyme level and growth rate. Addition of thymidine to the medium did not increase the growth rate of SW 403 cells, indicating that the slow growth of this cell line is probably unrelated to low dTMP synthetase levels.

Reduced binding of [^{3}H]FdUMP to dTMP synthetase, which would result in an underestimate of cellular enzyme content, might occur in the absence of adequate CH_2FH_4, a cofactor which is required for covalent complex formation between FdUMP and dTMP synthetase (4). The values for [^{3}H]FdUMP binding shown in Table 1 were determined using endogenous CH_2FH_4 as the cofactor. However, the same values were obtained when titrations were performed in the presence of exogenous CH_2FH_4 at a concentration of *60* μM, indicating that inadequate cofactor levels are not responsible for the observed variation in the levels of dTMP synthetase. Furthermore, titrations performed daily over several days of cell growth demonstrated no fluctuation in the level of dTMP synthetase present within the cells until the culture became visibly overgrown, at which time a marked decrease in the level of enzyme was observed. At no time during the growth curve was endogenous CH_2FH_4 present at a level below that required for maximal binding of FdUMP.

TABLE 1

FdUMP binding capacity of tumor cells

[^{3}H]FdUMP binding in each cell line was determined as described under Materials and Methods.

Cell line	[^{3}H]FdUMP bound	
	fmoles/10^6 cells	*fmoles/μg protein*
HuTu 80	72.1[a]	3.27
HT 29	7.2	0.26
SW 480	38.4	1.6
WIDR	17.6	0.58
SW 403	3.4	0.11

[a] Results are expressed as the mean of four determinations. The standard deviation in each case was less than 5%.

Cytotoxicity of FdUrd. We examined the possible effect of differing levels of dTMP synthetase on the cytotoxicity of the nucleoside analogue FdUrd in these cell lines. The sensitivity of the five tumor cell lines to FdUrd is shown in Fig. 2. The cell lines vary in their sensitivity to this compound, with EC_{50} values ranging from 0.44 nM for the SW 403 cell line to 16 nM for the HuTu 80 cell line. In all cases, the cytotoxic effects of FdUrd could be reversed by the addition of thymidine, indicating that FdUrd is exerting its toxic effect through inhibition of dTMP synthetase, as expected.

The correlation between cellular levels of dTMP synthetase and the concentration of FdUrd which is cytotoxic to a particular cell line is demonstrated in Fig. 3. The higher the level of dTMP synthetase in the cell, the more FdUrd is required to produce cytotoxicity (and the higher the EC_{50}). Linear regression analysis of the data produces the line shown, with a correlation coefficient of $r = 0.98$.

Sensitivity to MTX. There have been several reports in which the cytotoxicity of MTX to tissue culture cells has been related to cellular dTMP synthetase activity. In these studies, enzyme activity was varied within a cell line either by direct inhibition of dTMP synthetase using FdUrd (10) or by the use of mutants with altered enzyme levels (9, 28). The sensitivity to MTX of the tumor cell lines that we have described, which contain different levels of dTMP synthetase, is shown in Table 2. The cell lines vary in their sensitivity, with the EC_{50} for MTX cytotoxicity ranging from 16 nM for the HuTu 80 cells to 56 nM for the SW 403 cells. In the presence of 100 μM hypoxanthine, which relieves the antipurine effects of MTX, all of the EC_{50} values are shifted to higher MTX concentrations, but the relative sensitivities of the five cell lines are unaltered.

The relationship between the concentration of MTX required for cytotoxicity (in the presence of 100 μM hypoxanthine) and dTMP synthetase levels in each cell line is shown in Fig. 4. An excellent inverse correlation is observed ($r = -0.95$); the higher the level of dTMP synthetase in the cell, the lower the EC_{50} for MTX in that cell line.

[^{3}H]MTX transport and dihydrofolate reductase activity. The inverse correlation observed between the EC_{50} for MTX and the dTMP synthetase level in these tumor cell lines is excellent. However, other parameters can influence the cellular toxicity of MTX. Resistance to MTX has been related both to decreased transport (29) of the drug and to increased levels of the target enzyme,

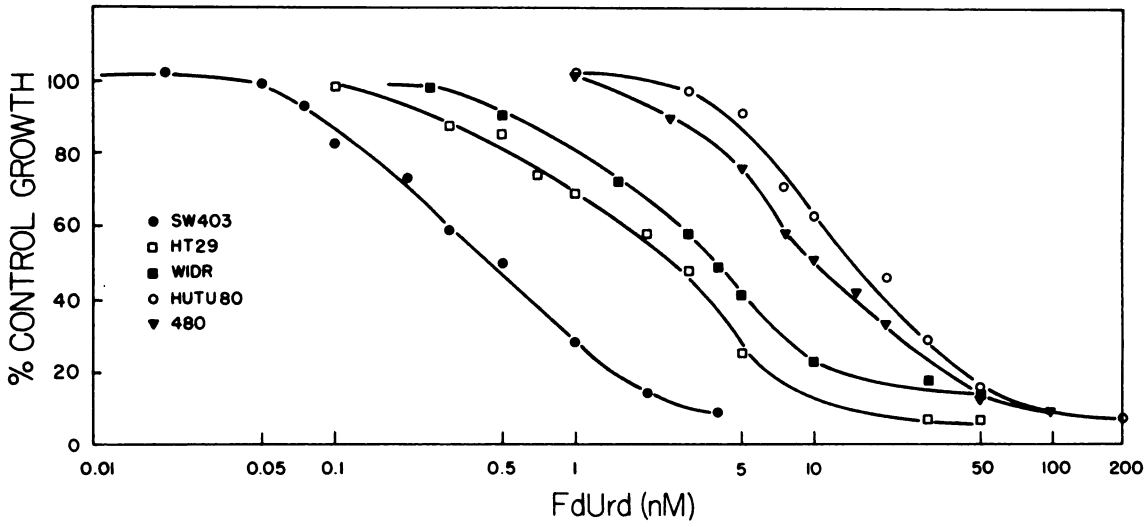

FIG. 2. *Effect of FdUrd on cell growth*

Cytotoxicity of FdUrd to SW 403 (●), HT 29 (□), WIDR (■), HuTu 80 (○), and SW 480 (▼) was determined as described under Materials and Methods. The results shown are those of a typical experiment.

dihydrofolate reductase (28). We therefore examined both of these factors in the tumor cell lines.

Rapid [^{3}H]MTX uptake was observed in all of the cell lines; data for three of the cell lines are shown in Fig. 5. In each case, uptake appeared to be linear during the first minutes of drug exposure, after which the rate of drug accumulation slowed and approached a steady state. These data are similar to those which have been reported for MTX uptake in other cell types (25, 26). The steady-state levels reached do not vary by more than 2-fold among the various cell lines. Efflux of the drug from all of the cell lines was equally rapid, with 60–75% of the drug lost within 20 min in all cases.

The possibility of variability in dihydrofolate reductase levels, the enzyme to which MTX binds, was also investigated. In Table 3 are listed the dihydrofolate reductase activities which were measured in cell sonicates. Among four of the five cell lines, almost no difference was seen in the activity of this enzyme. The SW 403 cell line, which is the least sensitive to MTX, contained about twice the enzyme activity of the other cell lines. Dihydrofolate reductase levels were also estimated by measuring the amount of non-exchangeable [^{3}H]MTX present in cells following a short incubation with this compound (Table 3). It has been demonstrated that this non-exchangeable drug fraction can represent between 95% and 99% of the intracellular dihydrofolate reductase (26). For both MTX transport and dihydrofolate reductase activity (or levels), no correlation with MTX cytotoxicity is apparent.

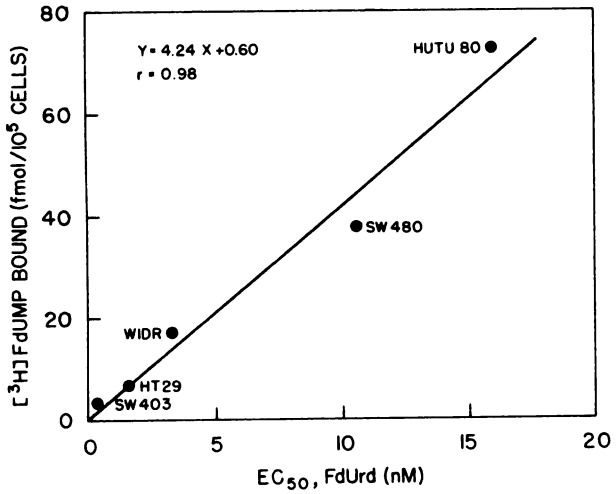

FIG. 3. *Correlation between dTMP synthetase level and FdUrd cytotoxicity*

The EC_{50} values for FdUrd were determined from curves such as those of Fig. 2. Each value represents the mean of four determinations. The standard deviation in all cases was less than 5%. dTMP synthetase levels (i.e., FdUMP binding sites) for each cell line are taken from Table 1.

DISCUSSION

In this study we have demonstrated an excellent correlation between intracellular levels of dTMP synthetase and cytotoxicity of two chemotherapeutic agents, FdUrd and MTX, to five independently derived human gastrointestinal tumor cell lines. Other parameters (i.e., drug

TABLE 2

Sensitivity of tumor cells to MTX

EC_{50} values for MTX in each cell line were determined as described under Materials and Methods.

Cell line	EC_{50} of MTX	
	Alone	+100 μM Hypoxanthine
	nM	*nM*
HuTu 80	16[a]	25
HT 29	38	60
SW 480	19	32
WIDR	31	50
SW 403	56	63

[a] Each value represents the average of four determinations. The standard deviation in each case was less than 5%.

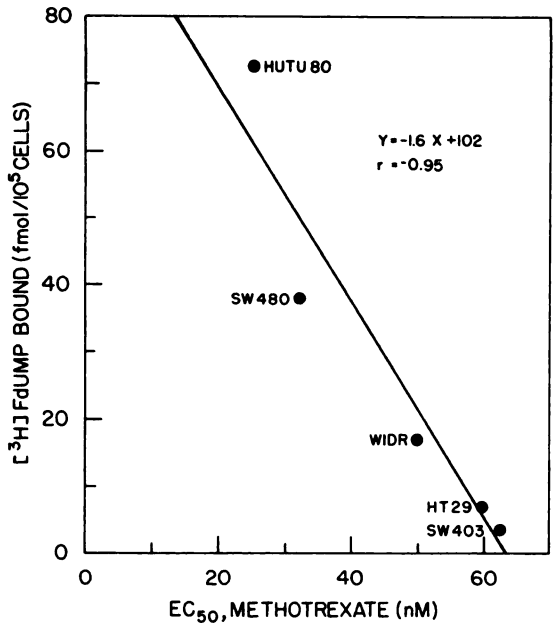

FIG. 4. *Correlation between MTX cytotoxicity and dTMP synthetase level*

The data used are taken from Tables 1 and 2. MTX cytotoxicity was determined in the presence of 100 μM hypoxanthine.

transport, dihydrofolate reductase activity, intracellular folate levels) which might influence the cytotoxicity of these drugs were also examined. Although some variations in these parameters were observed, no correlation between any of these other parameters and drug cytotoxicity was apparent.

The correlation between dTMP synthetase levels and the EC_{50} for FdUrd is a direct one: the more dTMP synthetase per cell, the greater the amount of FdUrd

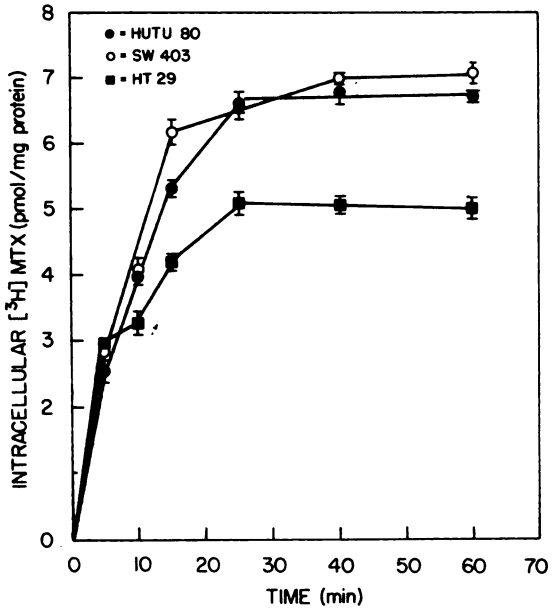

FIG. 5. *Time course of uptake of [^{3}H]MTX by tumor cells*

Data are shown for HuTu 80 (●), SW 403 (○), and HT 29 (■) cell lines. The external [^{3}H]MTX concentration was 1 μM. Experimental details are described under Materials and Methods.

TABLE 3

Dihydrofolate reductase activity in tumor cells

Activity of dihydrofolate reductase at 25° in cell sonicates was determined as described under Materials and Methods. Dihydrofolate levels are represented by the amount of non-exchangeable [^{3}H]MTX present within the cells.

Cell line	Dihydrofolate reductase	
	Activity	Level
	μM/hr/μg protein [a]	*μmoles/mg protein* [b]
HuTu 80	0.044 ± 0.099	1.4
HT 29	0.039 ± 0.003	1.3
SW 480	0.058 ± 0.003	1.8
WIDR	0.039 ± 0.005	1.4
SW 403	0.091 ± 0.005	2.7

[a] Results are expressed as the mean ± standard deviation of six determinations.

[b] Each value represents the average of four determinations.

required to inhibit cell growth by 50%. Because of the stoichiometric nature of the inhibition of dTMP synthetase by fluoropyrimidines (4), the amount of this enzyme present within the cell must ultimately determine the amount of FdUrd (in the form of the active metabolite FdUMP) required to inhibit completely the enzyme activity. However, several parameters may influence formation *in vivo* of the ternary complex between FdUMP, dTMP synthetase, and CH_2FH_4. Houghton *et al.* (21), in examining a series of xenografts of human colorectal adenocarcinomas, found no correlation between dTMP synthetase level and responsiveness to fluoropyrimidines. Rather, nonresponsive tumors appeared to lack sufficient endogenous CH_2FH_4 to allow maximal binding of FdUMP to dTMP synthetase. For the five cell lines examined in the current study, the amount of [^{3}H] FdUMP bound to dTMP synthetase in cell sonicates was the same whether measurements were made in the presence or absence of added CH_2FH_4. It would therefore appear that, in these cell lines, the cofactor is not limiting to ternary complex formation.

FdUrd cytotoxicity *in vivo* also requires generation of sufficient intracellular FdUMP from FdUrd. Either altered transport of this nucleoside (30) or reduced thymidine kinase activity (31), which is responsible for generation of FdUMP from FdUrd, could result in diminished cytotoxicity. When cells were fed [^{3}H]FdUrd, thus allowing generation of [^{3}H]FdUMP *in situ*, the values obtained for ternary complex formation were similar to those determined by direct titration for all cell lines (data not shown). This finding strongly suggests that neither impaired nucleoside transport nor reduced thymidine kinase activity can account for the observed variability in FdUrd sensitivity among these cell lines and that this variability is, in fact, a reflection of the differing levels of target enzyme.

The correlation between the EC_{50} for MTX and cellular dTMP synthetase levels is an inverse one: the less dTMP synthetase per cell, the more MTX required to inhibit cell growth by 50%. These results agree with other recent studies which have suggested that dTMP synthetase activity is crucial to the development of MTX cytotoxicity, since it is the enzyme responsible for depletion

of the reduced folate pool. When this activity is diminished, either artificially or by mutant selection, MTX cytotoxicity is reduced (9–11, 28).

Hypoxanthine should relieve the effect of depleted intracellular reduced folates on purine biosynthesis. The correlation demonstrated in Fig. 4 is for MTX cytotoxicity in the presence of hypoxanthine, which affords some protection against MTX cytotoxicity in all of the cell lines, although to varying extents (Table 2). These results more directly reflect the importance of dTMP synthetase activity to MTX cytotoxicity by removing any variability in the demand for reduced folates for purine biosynthesis which might exist among the cell lines. The cytotoxicity observed under these conditions should reflect the depletion of CH_2FH_4 due to dTMP synthetase activity and the effect of this depletion on further thymidylate synthesis. However, if one examines the correlation between the EC_{50} values for MTX alone with dTMP synthetase levels (data from Tables 1 and 2), one still observes a significant inverse correlation ($r = -0.7$).

We suggest that measurements of dTMP synthetase levels in tumor biopsies, by the methods we have employed (7) or by other published procedures (32), can be of potential importance in determining effective drug levels and/or therapeutic regimens and perhaps in reducing the degree to which tumor heterogeneity can render therapy less than maximally effective. High dTMP synthetase levels may indicate that fluoropyrimidine therapy would be unable to achieve the concentrations of drug *in vivo* necessary for cytotoxicity; however, such tumors might represent excellent targets for MTX therapy. Low dTMP synthetase levels would indicate a tumor of potentially good responsiveness to FdUrd but not to MTX. Furthermore, comparison of cellular dTMP synthetase levels measured by direct titration using [^{3}H] FdUMP with enzyme levels determined by exposing cells to [^{3}H]FdUrd will indicate whether other parameters (i.e., transport, kinase deficiencies, folate levels) might compromise the potential value of FdUrd for this tumor. The use of such measurements in conjuction with a clinical trial involving hepatic FdUrd infusion is currently being designed.

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Send reprint requests to: Dr. Wendy L. Washtien, Department of Pharmacology, Northwestern University Medical School, 303 E. Chicago Avenue, Chicago, Ill. 60611.