

Thymidylate Synthase Level as the Main Predictive Parameter for Sensitivity to 5-Fluorouracil, but not for Folate-based Thymidylate Synthase Inhibitors, in 13 Nonselected Colon Cancer Cell Lines¹

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ABSTRACT

Thymidylate synthase (TS), a critical enzyme in the *de novo* synthesis of thymidylate, is an important target for fluoropyrimidines and folate-based TS inhibitors. In a panel of 13 nonselected human colon cancer cell lines, we evaluated the role of TS levels in sensitivity to 5-fluorouracil (5FU) and four folate-based TS inhibitors that have been introduced recently into the clinic: ZD1694 (Tomudex, Raltitrexed, TDX), GW1843U89 (GW), LY231514 (LY), and AG337 (Thymitaq, AG). Because the latter compounds have different transport and polyglutamylation characteristics, we also related these parameters with drug sensitivity, measured by the sulforhodamine B assay after 72 h of drug exposure. For 5FU, the IC₅₀s varied from 0.8 to 43.0 μM. Leucovorin (LV) potentiated the activity of 5FU in only 4 of 13 cell lines. Sensitivity to folate-based TS inhibitors was variable; IC₅₀s were in the range of: 5.3–59.0 nM TDX; 11.0–1570 nM LY; and 0.5–8.9 nM GW. Eleven of 13 cell lines had an IC₅₀ for AG between 1.3 and 5.3 μM. Two cell lines were resistant to AG, Colo201 and SW1116, with IC₅₀s of 27 and 29 μM, respectively. TS catalytic activity (conversion of dUMP to dTMP) varied from 62 to 777 pmol/h/10⁶ cells. The number of FdUMP binding sites varied from 32 to 231 fmol/10⁶ cells. Regression analysis showed a significant relation between TS catalytic activity and IC₅₀s for 5FU and 5FU/LV. *K_is* for FdUMP showed a significant Spearman

rank correlation with the IC₅₀s of AG and GW. The role of antifolate transport, accumulation, and polyglutamylation was determined with [³H]methotrexate (MTX) as a reference compound. [³H]MTX influx via the reduced folate carrier varied from 18.6 to 150 fmol/10⁶ cells/min. Folylpolyglutamate synthetase (FPGS) activity showed a range from 47 to 429 pmol/10⁶ cells/h. A total of 24 h of [³H]MTX accumulation showed a 20-fold variation, from 1.2 to 21.8 pmol/10⁶ cells. FPGS levels showed a Spearman rank positive correlation with cytotoxicity to TDX.

In conclusion, in a heterogeneous nonselected human colon cancer cell line panel, the best predictor for sensitivity to 5FU and 5FU/LV was TS activity. Multiple sensitivity determinants were of importance for antifolate TS inhibitors, including FPGS activity and TS enzyme kinetics.

INTRODUCTION

5FU,³ in combination with LV, is still the standard treatment of advanced colorectal carcinoma (1) with response rates of 20–30% but with no major benefits in terms of increase in survival (2). Various 5FU regimens are beneficial for adjuvant treatment of patients with colorectal cancer (3). 5FU is a pro-drug, which after conversion to FdUMP inhibits TS (4). TS inhibition is an important mechanism of 5FU cytotoxicity, although 5FU is also incorporated into RNA and DNA. TS is an essential enzyme for the *de novo* synthesis of thymidylate (dTMP), a precursor for DNA synthesis, for which 5,10-methylene-tetrahydrofolate (5,10-CH₂-THF) is the methyl donor (4, 5). LV, a precursor for 5,10-CH₂-THF, is often combined with 5FU, resulting in enhanced cytotoxicity in model systems (6) and increased response in advanced colorectal cancer compared with similar 5FU regimens (7, 8).

Cellular sensitivity to 5FU is related to the rate of conversion to active metabolites, the amount of incorporation into RNA, and alterations at the TS level (*e.g.*, enhanced enzyme recovery and synthesis; 9). Besides these target-specific determinants of drug sensitivity, others have been described such as DNA repair activity, dUTPase levels, and effects on tumorsuppressors or oncogenes. As a consequence of the potential role of TS in nucleotide synthesis, this enzyme is considered to be an important target for drug therapy (6). Several new folate-based TS inhibitors have been developed and have subsequently been

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³ The abbreviations used are: 5FU, 5-fluorouracil; TS, thymidylate synthase; LV, leucovorin; 5,10-CH₂-THF, 5,10-methylene-tetrahydrofolate; MTX, methotrexate; RFC, reduced folate carrier; FPGS, folylpolyglutamate synthetase; HepBSS, HEPES buffered saline solution.

entered into clinical trials (10). For ZD1694 (Tomudex, Raltitrexed), Phase III trials revealed activity similar to some regimens of 5FU/LV (11). GW1843U89 (12), LY231514 (13), and AG337 (Thymitaq; 14) are presently in earlier phases of clinical development. These compounds have different properties concerning the efficiency of transport by the RFC and the capacity to be polyglutamylated. ZD1694, LY231514, and GW1843U89 display efficient cellular uptake via the RFC (15), and these compounds are also good substrates for FPGS, although GW1843U89 is usually converted only to its diglutamate form (16). In contrast to other antifolate TS inhibitors, GW1843U89 has a similar potency of TS inhibition as its monoglutamate and polyglutamate forms (16). Recently, LY231514 has been described as a multiple targeted antifolate, because it inhibits several folate-requiring enzymes including a potent inhibition of dihydrofolate reductase (17). AG337 is a lipophilic compound, which is transported by passive diffusion and is not polyglutamylated (18).

Preclinical data suggest a correlation between TS levels and sensitivity to 5FU. Beck *et al.* (19) reported that TS activities correlated with 5FU IC_{50} s in a panel of 19 nonselected breast, digestive tract, and head and neck cancer cell lines. Johnston *et al.* (20) also described a positive correlation between TS activity and 5FU sensitivity in 10 digestive tract and breast cancer cell lines, including several cell lines with acquired highly amplified TS expression. Several studies reported an association between tumor TS levels and response to chemotherapy in patients. Both low TS levels and a high extent of TS inhibition are predictive for response to 5FU treatment in patients with advanced colorectal cancer (21), whereas high intratumoral TS mRNA expression, measured by reverse transcription-PCR, was related to resistance to protracted 5FU infusion in patients with colorectal cancer (22) and in gastric cancer (23). The expression of TS, measured by immunohistochemical staining, is an important independent prognostic determinant of the disease-free and overall survival of patients with rectal cancer treated with adjuvant therapy (24). Other parameters, such as FPGS activity, may also be important for predicting the outcome of 5FU-based therapy (25–27).

Collectively, a large series of *in vitro* data have demonstrated predictive relationships between TS activity and sensitivity to TS inhibitors (mainly 5FU). However, it should be realized that these predictive parameters were limited to panels of cell lines with either different histological phenotypes or after provoking resistance to 5FU or a TS inhibitor. In these latter studies, multiple mechanisms of acquired resistance were described including induction of TS, disturbed RFC, or a decrease in FPGS activity (28–30).

In the present study, we used a panel of 13 human colon cancer cell lines, which were not selected for resistance to any of the used drugs. Moreover, the panel of cell lines differed in biological characteristics such as differentiation and primary tumor site. As such, this panel may be representative for a wide spectrum of colon cancers. Within this panel of cell lines, we have tested whether the sensitivity to 5FU and the new antifolate-based TS inhibitors was correlated with TS and other determinants for antifolate response, such as transport and polyglutamylation.

MATERIALS AND METHODS

Drugs and Chemicals

5FU was obtained from Sigma Chemical Co. (St. Louis, MO). The antifolates used in this study were generously provided by the following persons/institutions: ZD1694 (Tomudex, Raltitrexed; Dr. F. T. Boyle, Zeneca Pharmaceuticals, Macclesfield, United Kingdom), ZD1694 pentaglutamate (Dr. A. L. Jackman, Institute Cancer Research, Sutton, United Kingdom), GW1843U89 (Dr. R. Ferone, Glaxo/Wellcome, Research Triangle Park, NC), LY231514 (Dr. C. Shih, Lilly Research Laboratories, Indianapolis, IN), AG337 (Thymitaq; Dr. R. C. Jackson, Agouron Pharmaceuticals, San Diego, CA), and MTX-Glu₂ (Schricks Company, Jona, Switzerland). DMEM was purchased from Flow Laboratories (Irvine, Scotland) and FCS from Life Technologies, Inc. (Paisley, United Kingdom). The 96-well, flat-bottomed plates were purchased from Costar (Corning Costar, Cambridge, MA). [6-³H]FdUMP (MT-692; specific activity, 19 Ci/mmol) and [3',5',7-³H]MTX (MT-701; specific activity, 23 Ci/mmol) were obtained from Moravex Biochemicals, Inc. (Brea, CA), [5-³H]dUMP (TRK-287; specific activity, 19 Ci/mmol) from Amersham International (Buckinghamshire, UK), and L-[U-¹⁴C]glutamic acid (NEC-290E, 296 mCi/mmol) from DuPont NEN Research products (Boston, MA). DL-Tetrahydrofolic acid (Sigma Chemical Co.) was converted to 5,10-methylenetetrahydrofolic acid (5,10-CH₂-THF) by addition of formaldehyde (31). The liquid scintillation fluid Ultima Gold was obtained from Packard (Tilburg, the Netherlands). We used the Bio-Rad protein assay for protein determination (32). Unless otherwise specified, all other chemicals were of analytical grade and commercially available.

Cell Culture

A panel of 13 nonselected colon cancer cell lines was used (Table 1). Except for the SNU-C1, all cell lines were growing as monolayers. Most cell lines were cultured with 7.5% FCS in Dulbecco's culture medium; only the SNU-C4 cell line was cultured with 10% FCS. None of the cell lines were made resistant to any drug. Cell line characteristics and origins are depicted in Table 1. SNU-C1 and SNU-C4 were a kind gift of Dr. P. G. Johnston (at that time at the National Cancer Institute, Naval Branch, Bethesda, MD). All other cell lines were obtained from the American Type Culture Collection.

Growth Inhibition Studies

Cells in exponential growth phase were transferred to 96-well flat-bottomed plates. We assured that cells were in exponential growth phase during the whole culture period. The plating densities of the cell lines mentioned in Table 1 were adapted to their doubling time, varying from 3,000 to 10,000 cells/well: SW1398, 7,000; SW620, 8,000; HT29, 6,000; Colo320, 3,000; WiDr, 5,000; Colo201, 5,000; SW1116, 10,000; SNU-C1, 10,000; SNU-C4, 6,500; SW948, 7,000; LoVo, 5,000; Colo205, 5,000; and LS174T, 5,000. The cells were plated in 100 μ l of medium, and after 24 h (day 0), 100 μ l of drug containing medium with 5% FCS were added. Drug exposure time was 72 h (day 3). The drugs were tested in triplicate in different concentration ranges, usually 3–4 log, depending on the drug, with more concentrations in the area of

Table 1 Growth characteristics and origin of human colon carcinoma cell lines

Differentiation	Primary tumor site	Doubling time (h)	Origin
Well differentiated			
Colo201	Adenocarcinoma from ascites fluid	17.3 ± 0.5	ATCC ^a CCL 224
Lovo	Adenocarcinoma from metastatic nodule in left supraclavicular region	20.9 ± 0.8	ATCC CCL 229
HT29	Adenocarcinoma colon	23.4 ± 1.8	ATCC HTB 38
Moderately differentiated			
Colo205	Adenocarcinoma from ascites fluid	17.9 ± 1.7	ATCC CCL 222
SW1116	Adenocarcinoma colon, Dukes' A	31.9 ± 3.0	ATCC CCL 233
SNU-C1	Colon descendens	32.7 ± 5.2	Park <i>et al.</i> (33)
WiDr	Adenocarcinoma colon	16.7 ± 1.5	ATCC CCL 218
LS174T	Adenocarcinoma colon, Dukes' B	17.8 ± 1.3	ATCC CL 188
Poorly differentiated			
SNU-C4	Colon transversum	17.0 ± 0.4	Park <i>et al.</i> (33)
SW948	Adenocarcinoma colon	25.8 ± 2.6	ATCC CCL 237
SW620	Lymph node metastasis	25.2 ± 1.1	ATCC CCL 227
Colo320	Adenocarcinoma colon	22.2 ± 1.9	ATCC CCL 220
SW1398	Adenocarcinoma colon	22.8 ± 0.6	Rutzky (34)

^a ATCC, American Type Culture Collection.

the IC₅₀. Growth-inhibitory effects were evaluated with the standard sulforhodamine B by measurement of the absorbance on day 0 and day 3 cells (35). The IC₅₀ was the drug concentration at which cell growth is 50% inhibited based on the difference of absorbance values on day 0 and day 3 of drug exposure as interpolated from a graph in which growth inhibition is plotted against absorbance (36). For several of these cell lines, we used the median effect model (37) to calculate the sigmoidity (Hill coefficient) and calculate the *Dm* (equivalent to IC₅₀s). However, this approach did not provide additional information. In the concentration range used for these experiments, growth curves seldomly reached absorbance values below that of day 0.

TS Assays

Cells to be used for enzyme assays were harvested 2 days after passaging when they were in an exponential growth phase. Cells were trypsinized, washed, counted, and frozen as cell pellets in liquid nitrogen and subsequently stored at -80°C until enzyme analysis. Cell pellets could be stored for at least 6 months without loss of activity. Cell pellets were thawed by the addition of ice-cold assay buffer, sonicated (three times 5 s with intervals of 10 s), and subsequently centrifuged at 14,000 × *g* for 15 min at 4°C. The supernatant was used for enzyme assays.

FdUMP Binding Assay. The binding assay with [6-³H]-FdUMP as a ligand for determining the number of free FdUMP binding sites of TS was carried out as described previously (38). In brief, the reaction mixture contained 50 μl of enzyme suspension (equivalent to 1 × 10⁶ cells) from supernatants, 50 μl of 6.4 mM CH₂-THF, 15 μl of 570 nM [6-³H]-FdUMP (final specific activity, 19 Ci/mmol) in a total volume of 250 μl in Tris-HCl buffer (200 mM, pH 7.4). The reaction was started by adding the enzyme and was incubated at 37°C for 1 h and stopped by adding 500 μl of 10% activated charcoal (to remove free FdUMP). For each cell line, linearity of binding with respect to amount of enzyme was established. After centrifugation, radioactivity was estimated by liquid scintillation counting of 250 μl of supernatant.

TS Catalytic Assay. This assay determines the catalytic activity of TS by means of ³H release during the TS catalyzed

conversion of [5-³H]dUMP to dTMP (39). The TS catalytic activity was measured at a saturating substrate concentration (10 μM dUMP) and at the approximate half-saturating substrate concentration (1 μM dUMP). In brief, the assay consisted of 25 μl of enzyme suspension (in different dilutions) and 5 μl of 6.4 mM CH₂-THF in a total volume of 50 μl of TRIS-HCl buffer (pH 7.4). In the assays in which inhibition of TS was measured, 10 nM FdUMP, 2 μM ZD1694 pentaglutamate, or 50 μM LY231514 was added. These concentrations were based on inhibition experiments described earlier (40). The reaction was started by adding 10 μl [5-³H]dUMP (1 or 10 μM final concentration; final specific activity, 50 and 5 mCi/mol, respectively), incubated for 15–60 min at 37°C, and stopped by adding 50 μl of ice-cold 35% trichloroacetic acid and 250 μl of 10% neutral activated charcoal. For each cell line, linearity of 5-[³H]dUMP conversion with respect to amount of protein and time was established. After centrifugation, 150 μl of the supernatant were collected and counted by liquid scintillation.

Western Blot Analysis. The cells were harvested during the logarithmic growth phase, washed, and centrifuged; the pellet was immediately frozen in liquid nitrogen. Samples were kept at -80°C. The cytosolic fraction was isolated by lysis in buffer containing 0.1% Triton X-100, 150 mM NaCl, 10 mM Tris-HCl (pH 7.6), and 5 mM EDTA, followed by centrifugation for 10 min, 14,000 × *g* at 4°C. From each sample an equivalent amount of protein was processed by 12% SDS-PAGE, followed by electroblotting onto Hybond ECL nitrocellulose membranes. Human TS (partially purified from the TS-overexpressing human lymphoblastoid cell line, W1L2:C1, kindly provided by Dr. A. L. Jackman, Institute for Cancer Research, Sutton, United Kingdom; Ref. 41) was applied to each gel to verify the TS position on the gel. The membranes were, therefore, blocked overnight with blocking buffer [1% nonfat dry milk, 1% BSA, in 10 mM Tris-HCl (pH 8.0), 0.15 M NaCl, and 0.05% Tween 20], followed by incubation with the primary antibody, rabbit-anti-human TS (1:3000 dilution in blocking buffer, kindly provided by Dr. G. W. Aherne, Institute for Cancer Research, Sutton, United Kingdom; Ref. 42). Subsequently, the blot was incubated with the secondary antibody (1:1000 dilution), horseradish peroxidase-conjugated goat-anti-rabbit antibody. Finally,

the blot was stained using peroxidase-labeled luminol as the detection fluid for chemiluminescence (ECL kit; Amersham, Buckinghamshire, United Kingdom). The density of the X-ray bands was quantified using a densitometric scanner (GS690 imaging densitometer, Bio-Rad Laboratories S.A.-N.V., Nazareth, Belgium).

FPGS Assay

The FPGS assay was performed as described by Van der Wilt *et al.* (43) using aminopterin as the substrate. Previously, we demonstrated a good relation between this short-term FPGS activity assay, MTX polyglutamylation, and antifolate sensitivity (15, 44). Briefly, the cell pellets were resuspended in ice-cold Tris/HCl buffer (200 mM, pH 8.5) and supplemented with 30 mM NaHCO₃ and 45 mM β-mercaptoethanol at a concentration of 10–40 × 10⁶ cells/ml and lysed using sonication on ice (three times 5 s, with a 10-s interval), followed by centrifugation (14,000 × *g* for 15 min). FPGS activity was assayed in the presence of 1 mM [U-¹⁴C]L-glutamate (0.4 mCi/mmol), 5 mM MgATP, and 500 μM aminopterin (AMT) as the antifolate substrate. In this colon cancer cell line panel, the assay was linear between 1 and 3 h of incubation period. The reaction was terminated by heating the samples at 95°C for 3 min. After being chilled on ice, the samples were centrifuged to precipitate denatured proteins. Product and substrates were separated after spotting onto PEI-cellulose TLC sheets and chromatography, with 0.5% (w/v) NH₄Cl and 0.5% (v/v) β-mercaptoethanol acting as eluents. Spots of AMT-[¹⁴C]Glu₂ (*R_f* = 0) and unreacted [¹⁴C]Glu (*R_f* = 0.6) were cut out, and radioactivity was counted.

[³H]MTX Uptake

The [³H]MTX uptake was performed as described previously (45). In these experiments, the [³H]MTX uptake was used as a reference for the antifolate uptake in these cell lines, because it was shown that [³H]MTX uptake is predictive for (anti)folate transport (15). The cells were harvested in the logarithmic growth phase by trypsinization. Then they were resuspended in HepBSS containing 107 mM NaCl, 20 mM HEPES, 26.2 mM NaHCO₃, 5.3 mM KCl, 1.9 mM CaCl₂, 1.0 mM MgCl₂, and 7 mM D-glucose and adjusted to pH 7.4 with NaOH. Influx was determined over an incubation period of 3 min at 37°C with 2 μM [³H]MTX (0.5 Ci/mmol). Uptake of [³H]MTX at 4°C served as a control. Cells were centrifuged and washed with 10 ml of ice-cold HepBSS. The final cell pellet was resuspended in 0.5 ml water, and the amount of [³H]MTX was counted with liquid scintillation.

Polyglutamate Distribution and Total [³H]MTX Accumulation

The MTX polyglutamates were measured as described previously (44). We demonstrated previously that MTX polyglutamates are representative for antifolate polyglutamate accumulation (15). In brief, cells were plated at a density of 2.5 × 10⁶ cells per 10-cm² flask. After a recovery time of 24 h, cells were incubated for another 24 h with 1 μM [³H]MTX (0.25 Ci/mmol). Cells were washed in HepBSS harvested by trypsinization and suspended in 1 ml of HepBSS. Ninety μl

were used for measurement of the total [³H]MTX accumulation by liquid scintillation counting, 10 μl were used for cell counting, and 900 μl were pelleted and used for the analysis of the polyglutamate distribution after trichloroacetic acid extraction. The polyglutamate distribution was analyzed by anion exchange high-performance liquid chromatography. Separation was achieved using a PartiSphere Sax Column (internal diameter, 4.70 mm; length, 110 mm; pore size, 5 μm) obtained from Whatman Ltd. (Maidstone, United Kingdom). The high-performance liquid chromatography system consisted of a Gynkotek M480 gradient pump (Separations Analytical Instruments, H.I. Ambacht, the Netherlands) connected to a LC-75 UV-detector (Perkin-Elmer, Gouda, the Netherlands) and a flow-through radioactivity detector [Flo-One Beta (A200), Radiometric Instruments and Chemical Co., Inc., Tampa, FL]. A gradient for the separation with a flow rate of 1.0 ml/min was initiated after 4 min from buffer A (60 mmol/l NH₄H₂PO₄, pH 5.5) with 2% buffer B (600 mmol/l NH₄H₂PO₄, pH 5.5) to 100% buffer B for the next 16 min and back to 2% buffer B in the next 5 min. UV detection at 309 nm was used for identification. The chromatogram of radioactive polyglutamates was used to calculate the ratios between the different polyglutamates.

Statistical Analysis

Correlations between cytotoxicity results of 5FU and the antifolates and various parameters (TS activity assays and folate metabolism) were analyzed using Spearman rank correlations and linear regression using the SPSS 7.0 package. Although in this study multivariate analysis would seem logical to perform, the sample size (13 cell lines) is too small, and the number of drug sensitivity-related parameters (7) is too high to perform multivariate analysis.

In this study with 13 cell lines and 7 parameters for drug sensitivity to be analyzed, the level of significance is very much influenced by the multiple testing procedures. Using the Bonferroni correction for multiple testing, the real level of significance may be determined by the number of tests, which in our case would lead to an α of 0.05/7 = <0.01. However, to enable evaluation for each parameter we provide all significance levels at $P < 0.05$.

RESULTS

Growth Inhibition Studies

Growth inhibition varied according to the cell line and the drug tested; IC₅₀s for 5FU ranged from 0.8 μM (SNU-C1) to 43 μM (SW620) with no evidence of enhancement by LV in this 72-h exposure schedule in most cell lines (Table 2). There was no indication that drug sensitivity would be related to cellular proliferation. For ZD1694, IC₅₀s ranged from 5.3 nM (SNU-C4) to 59 nM (SW1116) in the nonselected cell lines. For GW1843U89, the lowest IC₅₀ was 0.5 nM (SW948 and SW1398), and the highest value was 8.9 nM (SW1116). LY231514 showed higher IC₅₀s compared with the other polyglutamatable folates ZD1694 or GW1843U89, with a range of 11.0 nM for WiDr to 1570 nM for the Colo201 cell line. The lipophilic compound AG337 showed high IC₅₀s in two cell lines, SW1116 and Colo201 of 29 and 27 μM, respectively. The

Table 2 Growth inhibition (IC₅₀) of TS inhibitors in colon cancer cell lines

IC₅₀s were calculated after 72-h drug exposure. LV was added in a final concentration of 5 μ M. Values are means \pm SE of three to six separate experiments.

cell line	5FU μ M	5FU/LV μ M	ZD1694 nM	LY231514 nM	GW1843 nM	AG337 μ M
SNU-C4	2.0 \pm 0.43	1.2 \pm 0.1	5.3 \pm 1.1	69.0 \pm 7.9	3.8 \pm 0.7	3.8 \pm 0.6
SNU-C1	0.8 \pm 0.4	1.0 \pm 0.4	11.1 \pm 3.9	96.0 \pm 3.4	2.0 \pm 0.6	4.6 \pm 0.7
LS174T	11.6 \pm 2.5	6.1 \pm 0.2	6.3 \pm 1.3	65.0 \pm 15	1.4 \pm 0.5	4.2 \pm 0.7
LoVo	3.0 \pm 1.0	3.0 \pm 1.3	6.5 \pm 1.4	79.0 \pm 6.5	1.3 \pm 0.1	3.0 \pm 0.7
SW1398	4.8 \pm 0.1	1.8 \pm 0.7	7.9 \pm 0.6	260.0 \pm 80.0	0.5 \pm 0.1	3.7 \pm 0.7
SW1116	4.8 \pm 0.3	5.5 \pm 0.8	59.0 \pm 11.3	123.0 \pm 13.0	8.9 \pm 2.7	29.0 \pm 3
SW948	4.7 \pm 0.7	2.1 \pm 0.5	6.9 \pm 2.2	79.0 \pm 6.4	0.5 \pm 0.02	1.3 \pm 0.2
SW620	43.0 \pm 13.3	44.0 \pm 13.3	37.0 \pm 10.5	360 \pm 127.2	3.2 \pm 0.8	3.8 \pm 0.8
Colo201	8.5 \pm 1.0	6.6 \pm 1.9	42.0 \pm 21.3	1570 \pm 45.0	7.6 \pm 1.6	27.0 \pm 2.5
Colo205	7.5 \pm 3.0	7.3 \pm 1.3	29.0 \pm 7.0	175.0 \pm 25.0	2.0 \pm 1.3	2.6 \pm 0.5
Colo320	4.0 \pm 0.8	3.3 \pm 0.4	48.0 \pm 20.8	230.0 \pm 90.0	4.8 \pm 0.8	5.3 \pm 0.8
HT29	16.0 \pm 5.0	9.4 \pm 0.3	26.0 \pm 1.2	330.0 \pm 40.0	2.6 \pm 0.5	3.3 \pm 0.4
WiDr	6.9 \pm 0.8	8.4 \pm 0.9	10.5 \pm 3.4	11.0 \pm 2.0	0.7 \pm 0.1	2.4 \pm 0.4

other cell lines had IC₅₀s for AG337 in a relatively small range (from 2.6 to 5.3 μ M).

GW1843U89 was consistently the most active compound among the antifolates, followed by ZD1694 and LY231514, whereas in all cell lines, the lipophilic nonpolyglutamatable AG337 was the least active compound. The sensitivity to 5FU and AG337 was very similar.

TS Levels

FdUMP Binding and TS Catalytic Assay. Both FdUMP, the metabolite of 5FU, and the new antifolates are targeted toward TS. TS levels measured by the number of FdUMP binding sites and the TS catalytic activity are depicted in Fig. 1. The values of the FdUMP binding showed a range from 36 (SW1398) to 230 fmol/10⁶ cells (Colo320).

A good correlation ($r = 0.84$, $P < 0.01$) was observed between the number of FdUMP binding sites and the TS catalytic activity (Fig. 2A) when all 13 cell lines were included in the analysis. When only the low range of TS levels was considered, the ratio between the two assays was, however, less pronounced ($r = 0.23$, $P = 0.23$), possibly because at this range not only the amount of TS protein (supposedly corresponding with the number of FdUMP binding sites), but also other (possibly kinetic) properties determine the specific enzyme activity. In general, the same cell lines showed either the highest or lowest TS levels using both the FdUMP binding and the TS catalytic activity assay.

To determine whether the extent of TS inhibition correlates with sensitivity to 5FU or the antifolates, we measured potential inhibition with the competitive (toward dUMP) TS inhibitor FdUMP (39), and with ZD1694 and LY231514, which are noncompetitive inhibitors toward dUMP (17, 46). Because it has been reported that TS mutations can affect dUMP and FdUMP binding, we determined their kinetics for TS. Competitive inhibition by FdUMP was comparable in most cell lines and as expected, higher at 1 μ M (80–85%) compared with 10 μ M dUMP (45–55%). Using Dixon plots for competitive inhibition, we calculated K_m s for dUMP and K_i values for FdUMP (Table 3). K_m s varied from 1.46 (SW1116) to 8.22 μ M (Colo205). The K_i s range from 0.82 nM

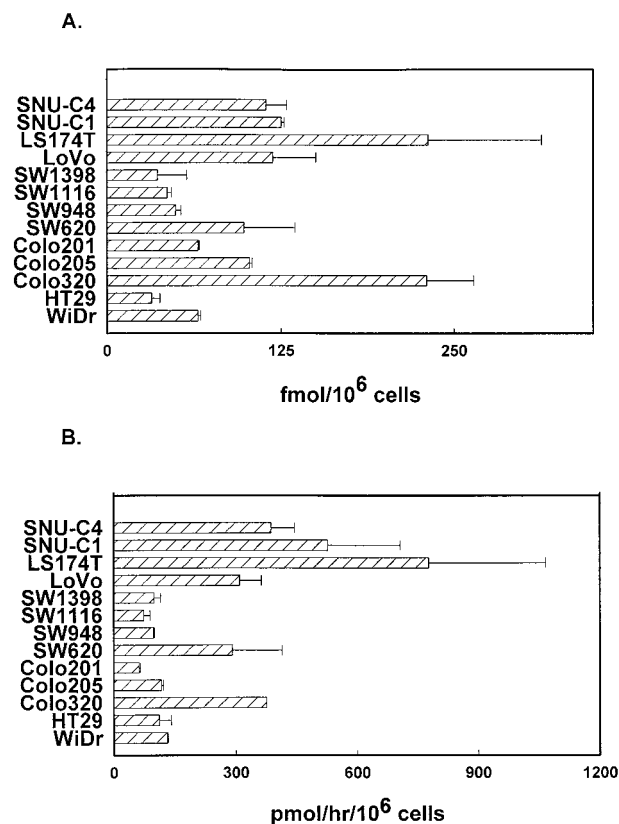


Fig. 1 TS enzyme levels in 13 nonselected colon cancer cell lines. FdUMP binding to TS (A) and TS catalytic activity (B) were measured with [³H]FdUMP and [³H]dUMP as substrates, respectively. Results are means of at least three separate experiments; bars, SE. Protein content was ~100 μ g/10⁶ cells for all cell lines.

(Colo320) to 2.41 nM (Colo201) with a median value of 1.27 (SNU-C4), demonstrating that FdUMP is a potent and consistent TS inhibitor in all cell lines. The noncompetitive TS inhibitors LY231514 and ZD1694 were tested at concentrations shown previously to cause 50% inhibition. This extent

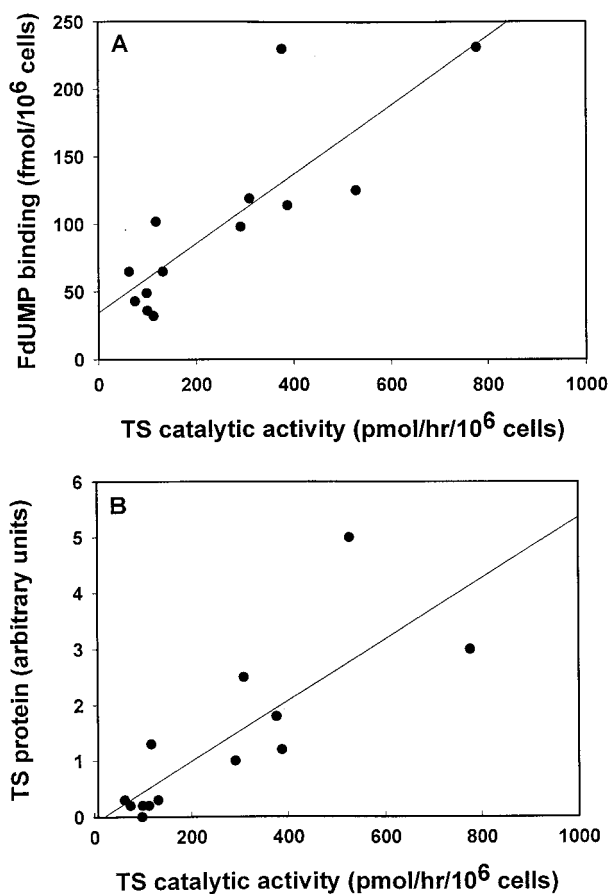


Fig. 2 Relation between various TS assays. In A, TS catalytic activity (in pmol/10⁶ cells/h) is plotted against FdUMP binding (in fmol/10⁶ cells; $r = 0.84$); in B, TS catalytic activity is plotted against TS protein (in arbitrary units; $r = 0.62$).

of inhibition is achieved at a lower concentration of ZD1694 than of LY231514 (Fig. 3). Inhibition of TS was consistently more pronounced at 10 μ M dUMP (60–70% inhibition for LY231514 and ZD1694) than at 1 μ M dUMP (45–50% for LY231514 and 38–55% for ZD1694) for both compounds, indicating that dUMP influences binding of (-anti) folates to the enzyme.

TS Western Immunoblotting. The results from the TS protein levels measured by Western immunoblotting are depicted in Fig. 2B. Two different patterns of protein expression can be distinguished. On the one hand, there is a group of 7 cell lines with a TS protein level in the range from 1 to 5 (Colo205, Colo320, SNU-C4, SNU-C1, SW620, LS174T, and LoVo cells). On the other hand, a very low TS protein level was measured in the other group of six cell lines, all with a very low relative TS protein level from 0 to 0.3 (SW1398, SW1116, SW948, WiDr, Colo201, and HT29 cells). A good correlation is seen when the TS protein levels are plotted against the TS catalytic activity levels (Fig. 2B).

[³H]MTX Influx

The growth-inhibitory activity of classical antifolates is dependent on its transport across the cell membrane by the RFC.

Table 3 Enzyme kinetic parameters for dUMP and FdUMP in colon cancer cell lines

Values are means and were calculated from three to five separate experiments using Lineweaver-Burk and Dixon plots. SE was <20%.

	K_m in μ M dUMP	K_i in nM FdUMP
SNU-C4	2.57	1.27
SNU-C1	2.04	1.07
LS174T	3.09	2.17
LoVo	1.62	1.22
SW1398	2.09	1.71
SW1116	1.46	1.02
SW948	2.52	1.24
SW620	7.5	1.91
Colo201	1.86	2.41
Colo205	8.22	1.1
Colo320	2.11	0.82
HT29	5.09	1.68
WiDr	2.85	1.06

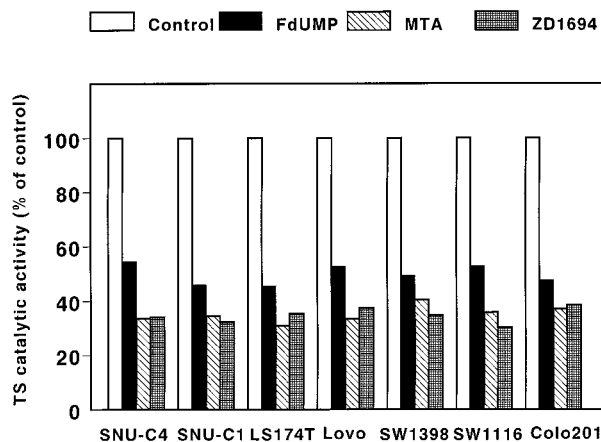


Fig. 3 TS inhibition in colon cancer cells. The antifolates were added simultaneously with [³H]dUMP (1 μ M) and a TS inhibitor (FdUMP, ZD1694 or LY231514) with concentrations of 10 nM, 2 μ M, or 50 μ M, respectively. For each experiment, control values were set at 100%, and relative inhibition was calculated. These values are given as the mean of at least three experiments; SE was <20%.

We demonstrated previously that [³H]MTX uptake is representative for the RFC activity (15); therefore, we used [³H]MTX to measure the rate of (anti)folate influx. The levels (Fig. 4) varied considerably from 19 (Colo320) to 150 fmol/10⁶ cells/min (SW1116). A subgroup of cell lines with a high folate transport level (>60 fmol/10⁶ cells/min) can be distinguished: LS174T, LoVo, SW1398, SW1116, and SW948 cell lines. The majority of the cell lines have a folate transport level from 19 to 51 fmol/10⁶ cells/min.

Polyglutamylation

FPGS Activity. Polyglutamylation of antifolates results in two distinct effects; polyglutamates are more effective TS inhibitors and provide an intracellular reservoir of antifolates. The activity of the enzyme catalyzing polyglutamylation, FPGS, showed a 10-fold difference in the cell line panel (Fig. 5), from

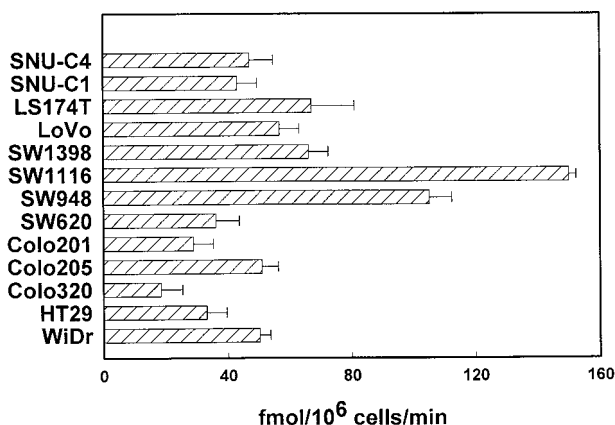


Fig. 4 Activity of the RFC as measured by $[3',5',7-^3\text{H}]\text{MTX}$ influx in colon cancer cells after incubation with $2\ \mu\text{M}$ MTX for 3 min at 37°C . The results are given as means of at least three separate experiments; bars, SE.

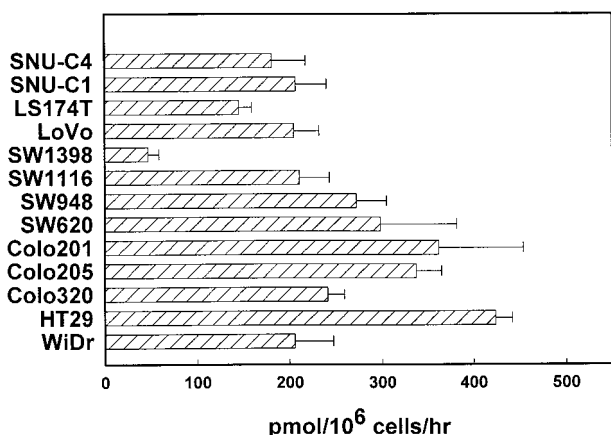


Fig. 5 FPGS activity in human colon cancer cell lines. Incubation was with $1\ \text{mM}$ $[^{14}\text{C}]\text{L}$ -glutamate and $500\ \mu\text{M}$ AMT as the substrate for FPGS for 1 h at 37°C . Values are means; bars, SE. The diglutamate was separated from glutamate using PEI-cellulose plates.

$47\ \text{pmol}$ of AMT-Glu₂/10⁶ cells/h (SW1398 cells) to $424\ \text{pmol}/10^6$ cells/h (HT29). Most cell lines had an FPGS activity in the range of 145 to $337\ \text{pmol}/10^6$ cells/h.

Total [³H]MTX Accumulation and Polyglutamate Distribution. TS inhibition by the classical antifolates is enhanced by polyglutamylation. Because MTX is a moderate substrate for FPGS, it provides a good estimate of the variability in polyglutamylation in different models. Such a variability subsequently also determines the differences in the extent of TS inhibition. Therefore, we determined both total MTX accumulation and polyglutamylation (Fig. 6). A large range in MTX accumulation was observed, with a low amount of [³H]MTX accumulation in the Colo201, SW1116, and HT29 cell lines (range from 1.15 to $2.8\ \text{pmol}/10^6$ cells/24 h) and much higher in the SW948, LS174T, SNU-C1, SNU-C4, and SW620 cell lines (range from 12 to $21.8\ \text{pmol}/10^6$ cells/24 h).

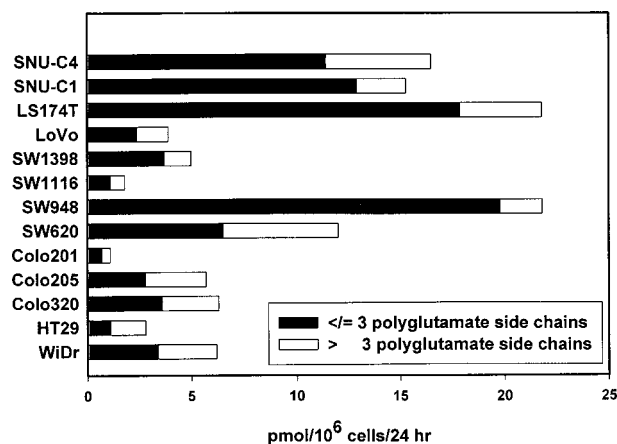


Fig. 6 Accumulation and polyglutamylation of $[3',5',7-^3\text{H}]\text{MTX}$ in colon cancer cells as measured after incubation with $1\ \mu\text{M}$ MTX for 24 h at 37°C . Results are depicted as the amount of MTX polyglutamates with ≤ 3 glutamate residues or > 3 glutamate residues. The values are means; SE was $< 30\%$.

Because the appearance of higher order polyglutamates is an indication of the efficacy of polyglutamylation, we calculated their distribution: ≤ 3 or more than three polyglutamate chains in relation to the absolute amounts (Fig. 6). SW620, HT29, and Colo205 cells accumulated a relatively high percentage of higher order polyglutamates (46, 62, and 51%, respectively), but SW948, LS174T, and SNU-C1 cells did not (only 9, 16, and 18%, respectively; Fig. 7). Interestingly, in cell lines with a high total 24 h MTX accumulation (SW948, LS174T, SW620, and SNU-C1), higher order polyglutamates made a smaller contribution to the total.

Relationship of TS and Folate Characteristics with Sensitivity

The aim of this study was to determine whether sensitivity to 5FU and TS inhibitors in a panel of nonselected colon cancer cell lines would be predictable by TS levels and/or other parameters known to be important in 5FU and antifolate sensitivity. For that purpose, we correlated the sensitivity to 5FU and the TS inhibitors with these parameters, using various statistical methods. The Spearman rank correlation was used because of the small population number and because the IC_{50} data were not distributed normally. To see whether the different parameters for TS and antifolate sensitivity were able to predict 5FU and antifolate sensitivity in this heterogeneous cell line panel, linear regression was used with the IC_{50} data transformed to the natural logarithm (Table 4).

TS Levels and Drug Sensitivity. A significant relationship between the sensitivity to both 5FU and 5FU/LV and the TS catalytic activity was observed with correlation coefficients of $r = 0.68$ ($P = 0.01$) and of $r = 0.62$ ($P = 0.02$), respectively. For the antifolate sensitivity, no such correlation was found.

When enzyme kinetic parameters of TS were used for evaluation, it is of interest to note that a high K_i for FdUMP was correlated with low sensitivity to GW1843U89 and AG337 with $r = -0.58$ ($P < 0.05$) and $r = 0.91$ ($P < 0.01$), respectively

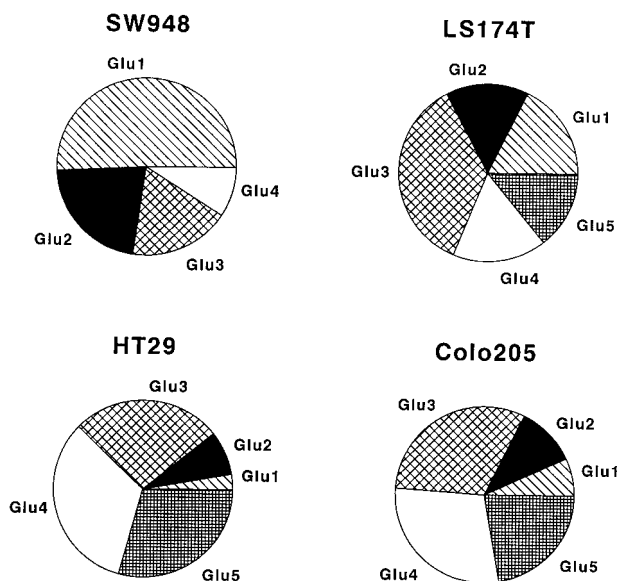


Fig. 7 Representative polyglutamylation patterns of several of colon cancer cell lines. The amounts of Glu₁-Glu₅ are given in percentages of all of the MTX-polyglutamates formed after incubation with [³H]MTX for 24 h at 37°C.

(Fig. 8). Probably, other factors than TS activity levels play an important role in antifolate sensitivity.

[³H]MTX Transport and Drug Sensitivity. When using MTX as the reference compound for folate transport, we observed that the antifolate resistant Colo320 cells had a low [³H]MTX influx level. High MTX transport in the SW1116 cells was, however, not associated with increased antifolate sensitivity. No correlations or predictive value between [³H]MTX influx and drug sensitivity could be found in these colon cancer cell lines. From these results, we concluded that factors beyond transport most likely determine antifolate sensitivity, *i.e.*, accumulation and polyglutamylation.

FPGS and Sensitivity. The positive correlation between FPGS and sensitivity was remarkable (Fig. 9). For example, cell lines that were more sensitive to LY231514 (SNU-C1, SNU-C4) had a relatively low FPGS activity. This resulted in an unexpected positive correlation for all cell lines, when a Spearman rank correlation was calculated. A positive correlation between FPGS and sensitivity to ZD1694 was found ($r = 0.64$, $P < 0.05$). Thus, the linear regression and multivariate analysis also showed that FPGS activity is of predictive value for ZD1694 sensitivity in this cell line panel ($r = 0.56$, $P = < 0.05$). Correlation coefficients were relatively high for the polyglutamatable antifolates LY231514 and GW1843U89. As anticipated, the correlation coefficient for the lipophilic nonpolyglutamatable AG337 was poor.

24-h MTX Polyglutamylation Related to Sensitivity. It may be expected that the accumulation of MTX polyglutamates after 24 h is a process, depending both on transport and FPGS activity. Despite large variations in polyglutamylation in these cell lines, the 24-h MTX polyglutamylation was not predictive for 5FU or 5FU/LV nor for antifolate sensitivity.

DISCUSSION

In this paper, we describe an important role for TS and folate enzymes in drug sensitivity in a panel of nonselected human colon cancer cell lines. Within this panel, drug sensitivity to 5FU or 5FU/LV was largely dependent on the TS activity, whereas for the antifolates tested, no common or logical measure of transport, drug accumulation, or TS kinetics was predictive of sensitivity. These data provide evidence that sensitivity of colon cancer cell lines to 5FU is determined by different parameters than those for the folate-based TS inhibitors.

FdUMP binding levels have often been associated with 5FU sensitivity of cell lines. Results were sometimes confusing because small numbers of cell lines and cell lines with induced resistance or with different histological origin were used. In this respect, Johnston *et al.* (20) reported a highly significant correlation ($r^2 = 0.94$, $P = 0.01$) between IC₅₀s for 5FU and FdUMP binding levels in five colon cancer cell lines, including two resistant lines (NCI H630-R1 and -R10) and three unselected lines (HCT 116, SNU-C1, and SNU-C4). Recalculation of the correlation by omission of the two 5FU-resistant H630 cell lines reduced the correlation coefficient to $r^2 = 0.64$ ($P = 0.2$). In a heterogeneous panel of 19 human tumor cell lines, including breast, digestive tract, and head and neck cell lines, Beck *et al.* (19) observed a weak correlation of $r^2 = 0.22$ ($P = 0.042$) between TS catalytic activity and log (10) IC₅₀s of 5FU. In the four colon cancer cell lines (WiDr, Colo1, Colo2, and Colo4), the correlation was no longer observed ($r^2 = 0.4$, $P = 0.6$; 19). Although these recalculated values should be considered carefully, they indicate the influence of the use of cell lines with acquired resistance or various tumor types for the outcome of this type of correlation study. In the present study, TS catalytic activity was significantly correlated with 5FU and 5FU/LV sensitivity in a panel comprising 13 human colon cancer cell lines. In addition to TS levels measured in other studies (19, 20), either as FdUMP binding or as TS catalytic activity, we also determined various enzyme kinetic parameters of TS and potential TS inhibition (21). However, only TS catalytic activity correlated with 5FU sensitivity. In cell lines with induced resistance, a good correlation was found between the TS catalytic activity and FdUMP binding (20). In our panel of nonselected colon cancer cell lines, TS catalytic activity also correlated with FdUMP binding assay, but only TS catalytic activity, the conversion of dUMP to dTMP, was predictive for 5FU and 5FU/LV sensitivity. This indicates that the level of physiological TS reaction is of major importance for 5FU sensitivity. The lack of a correlation of FdUMP binding drug sensitivity indicates that the extent of FdUMP binding is not only dependent on the level of TS but also on, for example, kinetic properties of TS.

It was reported previously that acquired 5FU resistance may be associated with FPGS (47). In 5FU-resistant cells, a decreased FPGS activity resulted in a lower amount of 5,10-CH₂-THF polyglutamates, which lowers the efficacy of TS/FdUMP/5,10-CH₂-THF ternary complex formation. However, in contrast to literature reports on acquired resistance to antifolates (30, 48), the present study focusing on cell lines with a natural difference in drug sensitivity suggests that a high FPGS activity was associated with a low sensitivity to ZD1694 but certainly not an increased sensitivity. To explain this apparent

Table 4 Correlation of IC₅₀ of TS inhibitors with determinants of drug sensitivity to TS inhibitors

In this table, the coefficients from the Spearman Rank analysis and the linear regression are depicted. The drug sensitivity in IC₅₀ is correlated with the various drug sensitivity parameters. Each correlation consists of a correlation coefficient (*r* (upper line) and the corresponding *P* (lower line), indicated at the right side of the table. Significant correlations are depicted in **boldface**.

	FdUMP	TS cat	<i>K_m</i>	<i>K_i</i>	Transport	FPGS	MTXaccum
Spearman rank correlation							
5FU	-0.38	-0.46	-0.03	0.06	-0.28	0.43	0.24 r
	0.21	0.11	0.92	0.85	0.36	0.14	0.43 P
5FU/LV	-0.39	-0.44	0.17	0.22	-0.42	0.54	0.18
	0.19	0.13	0.59	0.47	0.16	0.06	0.55
ZD1694		-0.2	0.27	-0.47	-0.19	-0.64	0.32
		0.52	0.37	0.1	0.54	0.02	0.29
GW1843U89		0.19	-0.02	-0.58	-0.28	0.43	-0.02
		0.55	0.96	0.04	0.35	0.14	0.95
LY231514		0.07	-0.1	-0.32	-0.1	0.47	-0.04
		0.81	0.75	0.28	0.74	0.11	0.9
AG337		0.25	-0.35	-0.91	0.11	0.06	-0.2
		0.41	0.24	0	0.71	0.84	0.51
Linear regression							
5FU	0.51	0.68	0.05	0.04	0.26	0.44	0.17 r
	0.07	0.01	0.87	0.9	0.4	0.13	0.59 P
5FU/LV	0.42	0.62	0.02	0.09	0.33	0.5	0.05
	0.15	0.02	0.95	0.78	0.27	0.08	0.87
ZD1694		0.22	0.07	0.34	0.18	0.56	0.28
		0.47	0.82	0.26	0.55	0.046	0.35
GW1843U89		0.19	0.23	0.48	0.25	0.42	0.06
		0.53	0.44	0.1	0.4	0.15	0.85
LY231514		0.03	0.01	0.57	0.02	0.47	0.22
		0.91	0.97	0.04	0.95	0.11	0.48
AG337		0.09	0.41	0.57	0.25	0.11	0.05
		0.77	0.16	0.04	0.42	0.73	0.88

disparity, it should be recognized that a markedly reduced sensitivity (>100-fold) to ZD1694 is generally observed in resistant cell lines that display <10% residual FPGS activity as compared with wild-type cells (28–30). However, as an exception to this, McGuire *et al.* (48) reported that human leukemia cells with 1% residual FPGS activity were <10-fold resistant to ZD1694. In the present study, we observed an 8-fold range in FPGS activity in the panel of colon cancer cell lines. This suggests that factors other than FPGS activity *per se* may play an additional role in determining the ZD1694 sensitivity profile in the panel of colon cell lines. Recent reports from our laboratory (49, 50) and others (51, 52) indicated that alterations in intracellular folate homeostasis can influence the polyglutamylation status of ZD1694. In particular, a subline in CEM leukemia cells with wild-type FPGS levels, comparable with the highest range of FPGS activity in the colon cancer cell lines, was 1000-fold resistant to ZD1694 as a result of a 7-fold increase in intracellular folate pool. The expanded folate pools abolished polyglutamylation of ZD1694, thereby abrogating its biological activity (49, 50). These results may imply that upon a culture of colon cancer cell lines in DMEM medium, containing supraphysiological concentrations of folic acid (8.8 μM), relatively minor alterations in folate homeostasis can reveal differences in ZD1694 sensitivity within a panel of colon cell lines with a ZD1694 sensitivity phenotype. These data also indicate that a high folate status may preclude activation of antifolates to active polyglutamates, whereas a low folate status may be implicated in antifolate toxicity in patients (53).

*K_i*s for FdUMP were determined because of the likelihood

of a relation with 5FU sensitivity. However, probably because the stability of the binding of FdUMP to TS is more dependent on the rate of dissociation of the complex, the *K_i*s did not correlate with sensitivity. However, these *K_i*s correlated with GW1843U89 and AG337 sensitivity, which suggests that enzyme kinetic properties of TS are important for the sensitivity to antifolates, which bind reversibly to TS. The basis for this unexpected association may be that these antifolates affect the nucleotide binding site of TS (54). This mechanism is suggested by the finding that AG337 could displace dUMP and FdUMP from purified human TS in a concentration-dependent manner (54). GW1843U89 almost completely prevented binding of FdUMP to TS at low concentration. Binding of the antifolates to TS might cause allosteric interactions between the TS subunits, changing the nucleotide binding. These data are suggestive for a correlation between kinetic parameters and sensitivity to antifolates. In this study, short-term measurements (³H]release assay and FdUMP binding assay) are compared with long-term incubations (growth inhibition). Because kinetic properties may have a different impact between short- and long-term experiments, the value of these kinetic associations warrants further exploration. Stout and Stroud (55) reported that GW1843U89 was a competitive inhibitor (at the folate binding site) and a noncompetitive inhibitor at the dUMP binding site using *Escherichia coli*-purified TS, suggesting a relation between the folate and the pyrimidine binding site of TS. The various mechanisms of inhibition of TS with both allosteric and nonallosteric interactions deserve further investigations and may elucidate more about inhibition characteristics of antifolates and FdUMP.

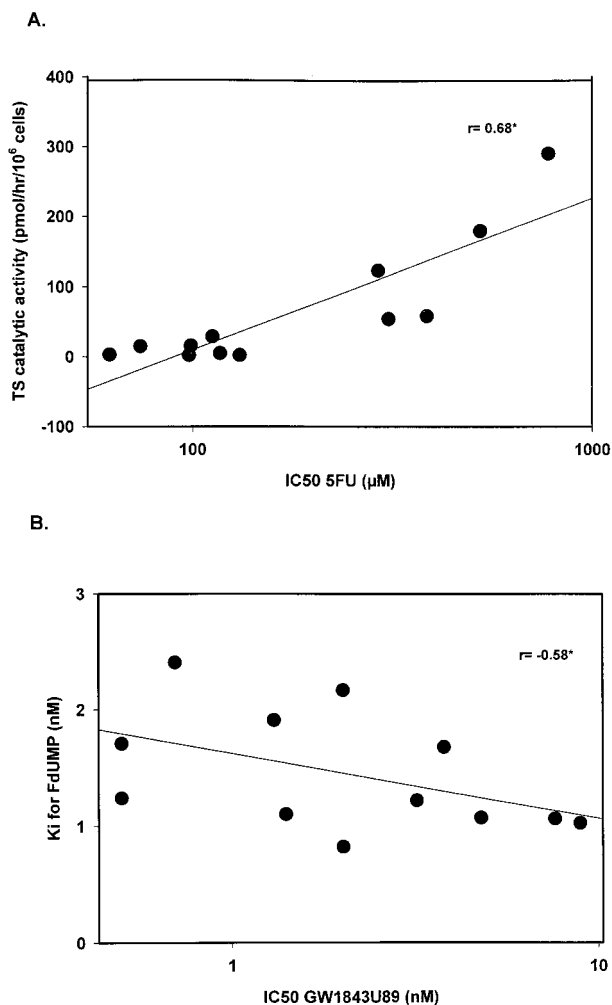


Fig. 8 Correlation plots and coefficients of the IC₅₀ of 5FU against TS catalytic activity (A) and the IC₅₀ of GW1843U89 against Ki for FdUMP (B). Significance is indicated by * when $P < 0.05$.

Several mechanisms of acquired resistance to folate-based TS inhibitors have been postulated, including increased TS (30), reduced transport, and polyglutamylation defects (15, 28–30). From our present results in the nonselected cell lines, none of these mechanisms *per se* appear to underlie a diminished drug sensitivity of one or more of the drugs tested. This seems to hold for both polyglutamatable and nonpolyglutamatable antifolate drugs. Altogether, the 5FU/antifolate drug sensitivity/resistance profile in the colon cancer cell lines is likely to have a multifactorial origin, which is based on a variability in folate-metabolizing enzymes, target enzymes, and differential downstream effects after target enzyme inhibition.

When we try to translate these results to *in vivo* sensitivity and clinical response to folate-based TS inhibitors, it seems likely that response will be determined either by a combination of parameters or by extremes, *e.g.*, a tumor with a very high TS is unlikely to respond to therapy with 5FU (21). Only for the expression of TS, measured either by enzyme assays (21), immunohistochemistry (24, 56), or reverse transcription-PCR

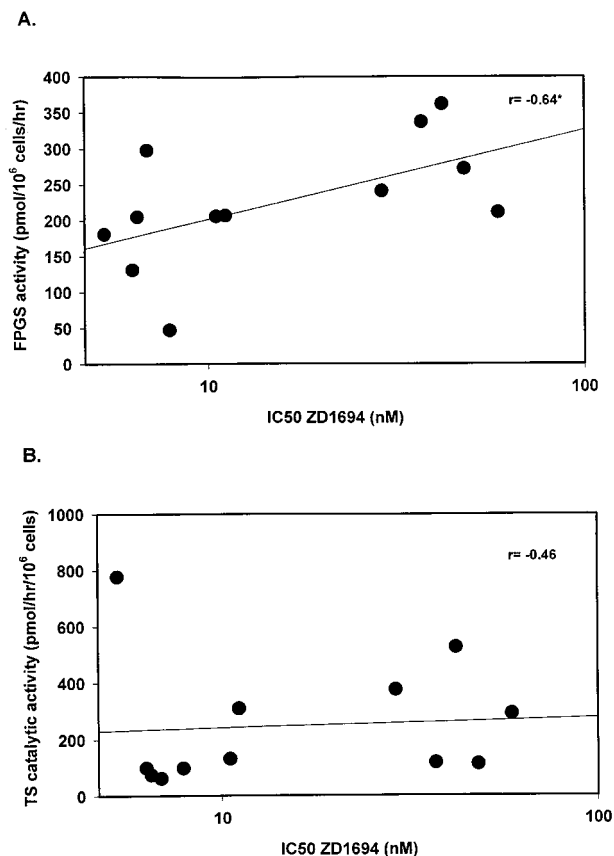


Fig. 9 Correlation plots and coefficients of IC₅₀ of ZD1694 against FPGS activity and the IC₅₀ of ZD1694 against TS catalytic activity (B). Significance is indicated by * when $P < 0.05$.

(22, 23), convincing retrospective data are available for an association between TS activity and response to 5FU-containing regimen. For FPGS activity, there is a weak suggestion of a correlation between FPGS activity and response to 5FU/LV (26). Data collection on TS and FPGS in ZD1694-treated tumors has just begun (57). Prospective trials in cancer patients should be performed to determine the differential clinical relevance of each possible resistance mechanism.

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REFERENCES

1. Moertel, C. G. Chemotherapy for colorectal cancer. *N. Engl. J. Med.*, 326: 1136–1142, 1994.
2. Advanced Colorectal Cancer Meta-analysis Project. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rates. *J. Clin. Oncol.*, 10: 869–903, 1992.
3. Haller, D. G., An overview of adjuvant therapy for colorectal cancer. *Eur. J. Cancer*, 31A: 1255–1263, 1995.
4. Pinedo, H. M., and Peters, G. J. Fluorouracil: biochemistry and pharmacology. *J. Clin. Oncol.*, 6: 1653–1664, 1988.

5. Houghton, J. A., Williams, L. G., De Graaf, S. S. N., Cheshire, P. J., Rodman, J. H., Maneval, D. C., Wainer, I. W., Jadaud, P., and Houghton, P. J. Relationship between dose rate of [6RS]leucovorin administration, plasma concentrations of reduced folates, and pools of 5,10-methylenetetrahydrofolates and tetrahydrofolates in human colon adenocarcinoma xenografts. *Cancer Res.*, 50: 3493–3502, 1990.
6. Van der Wilt, C. L., and Peters, G. J. New targets for pyrimidine antimetabolites for the treatment of solid tumors. I. Thymidylate synthase. *Pharm. World Sci.*, 16: 84–103, 1994.
7. Arbut, S. G. Overview of clinical trials using 5-fluorouracil and leucovorin for the treatment of colorectal cancer. *Cancer (Phila.)*, 6: 1036–1047, 1989.
8. Labianca, R., Pessi, A., Facendola, G., Pirovano, M., and Luporini, G. Modulated 5-fluorouracil (5-FU) regimens in advanced colorectal cancer: a critical review of comparative studies. *Eur. J. Cancer*, 32A (Suppl. 5): S7–S12, 1996.
9. Peters, G. J., and Jansen, G. Resistance to antimetabolites. In: R. L. Schilsky, G. A. Milano, and M. J. Ratain (eds.), *Principles of Antineoplastic Drug Development and Pharmacology*, pp. 543–585. New York: Marcel Dekker, Inc., 1996.
10. Rustum, Y. M., Harstrick, A., Cao, S., Vanhoef, U., Yin, M.-B., Wilke, H., and Seeber, S. Thymidylate synthase inhibitors in cancer therapy: direct and indirect inhibitors. *J. Clin. Oncol.*, 15: 389–400, 1997.
11. Cunningham, D., Zalberg, J. R., Rath, U., Olver, I., Van Cutsem, E., Svensson, C., Seitz, J. F., Harper, P., Kerr, D., Perez-Manga, G. Azub, M., Seymour, L., Lowerg, K., and the “Tomudex” Colorectal Cancer Study Group. (ZD1694): results of a randomised trial in advanced colorectal cancer demonstrate efficacy and reduced mucositis and leucopenia. The “Tomudex” Colorectal Cancer Study Group. *Eur. J. Cancer*, 31A: 1945–1954, 1995.
12. Duch, D. S., Banks, S., Dev, I. K., Dickerson, S. H., Ferone, R., Heath, L. S., Humphreys, J., Knick, V., Pendergast, W., Singer, S., Smith, G. K., Waters, K., and Wilson, H. R. Biochemical and cellular pharmacology of 1843U89, a novel benzoquinazoline inhibitor of thymidylate synthase. *Cancer Res.*, 53: 810–818, 1993.
13. Rinaldi, D. A., Burris, H. A., Dorr, F. A., Woodworth, J. R., Kuhn, J. G., Eckardt, J. R., Rodriguez, G., Corso, S. W., Fields, S. M., and Langley, C. Initial phase I evaluation of the novel thymidylate synthase inhibitor, LY231514, using the modified continual reassessment method for dose escalation. *J. Clin. Oncol.*, 13: 2842–2850, 1995.
14. Rafi, I., Taylor, G. A., Calvete, J. A., Boddy, A. V., Balmanno, K., Bailey, N., Lind, M., Calvert, A. H., Webber, S., Jackson, R. C., Johnston, A., Clendenin, N., and Newell, D. R. Clinical pharmacokinetic and pharmacodynamic studies with the nonclassical antifolate thymidylate synthase inhibitor 3,4-dihydro-2-amino-6-methyl-4-oxo-5-(4-pyridolthio)-quinazolinone dihydrochloride (AG337) given by 24-hour continuous intravenous infusion. *Clin. Cancer Res.*, 1: 1275–1284, 1995.
15. Westerhof, G. R., Schornagel, J. H., Kathmann, I., Jackman, A. L., Rosowsky, A., Forsch, R. A., Hynes, J. B., Boyle, F. T., Peters, G. J., Pinedo, H. M., and Jansen, G. Carrier- and receptor-mediated transport of folate antagonists targeting folate-dependent enzymes: correlates of molecular structure and biological activity. *Mol. Pharmacol.*, 48: 459–471, 1995.
16. Hanlon, M. H., and Ferone, R. *In vitro* uptake, anabolism, and cellular retention of 1843U89 and other benzoquinazoline inhibitors of thymidylate synthase. *Cancer Res.*, 56: 3301–3306, 1996.
17. Shih, C., Chen, V. J., Gossett, L. S., Gates, S. B., MacKellar, W. C., Habeck, L. L., Shackelford, K. A., Mendelsohn, L. G., Soose, D. J., Patel, V. F., Andis, S. L., Bewley, J. R., Rayl, E. A., Moroson, B. A., Beardsley, G. P., Kohler, W., Ratnam, M., and Schultz, R. M. LY231514, a pyrrolo[2,3-d]pyrimidine-based antifolate that inhibits multiple folate-requiring enzymes. *Cancer Res.*, 57: 1116–1123, 1997.
18. Webber, S., Bartlett, C. A., Boritzki, T. J., Hillard, J. A., Howland, E. F., Johnston, A. L., Kosa, M., Margosiak, S. A., Morse, C. A., and Shetty, B. V. AG337, a novel lipophilic thymidylate synthase inhibitor: *in vitro* and *in vivo* preclinical studies. *Cancer Chemother. Pharmacol.*, 37: 509–517, 1996.
19. Beck, A., Etienne, M. C., Chradame, S., Fischel, J. L., Formento, P., Rene, N., and Milano, G. A role for dihydropyrimidine dehydrogenase and thymidylate synthase in tumor sensitivity to fluorouracil. *Eur. J. Cancer*, 10: 1408–1411, 1994.
20. Johnston, P. G., Drake, J. C., Trepel, J., and Allegra, C. J. Immunological quantitation of thymidylate synthase using the monoclonal antibody TS 106 in 5-fluorouracil-sensitive and -resistant human cancer cell lines. *Cancer Res.*, 52: 4306–4312, 1992.
21. Peters, G. J., Van der Wilt, C. L., Van Groeningen, C. J., Smid, K., Meijer, S., and Pinedo, H. M. Thymidylate synthase inhibition after administration of fluorouracil with or without leucovorin in colon cancer patients: implications for treatment with fluorouracil. *J. Clin. Oncol.*, 12: 2035–2042, 1994.
22. Leichman, L., Lenz, H.-J., Leichman, C. G., Groshen, S., Danenberg, K., Baranda, J., Spears, C. P., Boswell, W., Silberman, H., Ortega, A., Stain, S., Beart, R., and Danenberg, P. Quantitation of intratumoral thymidylate synthase expression predicts for resistance to protracted infusion of 5-fluorouracil and weekly leucovorin in disseminated colorectal cancers: preliminary report from an ongoing trial. *Eur. J. Cancer*, 31: 1306–1310, 1995.
23. Lenz, H.-J., Leichman, C. G., Danenberg, K. D., Danenberg, P. V., Groshen, S., Cohen, H., Crookes, P., Silberman, H., Baranda, J., Garcia, Y., Li, J., and Leichman, L. Thymidylate synthase mRNA level in adenocarcinoma of the stomach: a predictor for primary tumor response and overall survival. *J. Clin. Oncol.*, 14: 176–182, 1996.
24. Johnston, P. G., Fisher, E. R., Rockette, H. E., Fisher, B., Wolmark, N., Drake, J. C., Chabner, B. A., and Allegra, C. J. The role of thymidylate synthase expression in prognosis and outcome of adjuvant chemotherapy in patients with rectal cancer. *J. Clin. Oncol.*, 12: 2640–2647, 1994.
25. Fischel, J. L., Chazal, M., Chéradame, S., Formento, P., Renee, N., Formento, J. L., Francoual, M., and Milano, G. Biochemical determinants for relative tumor sensitivity to Raltitrexed (Tomudex, TX) and 5-fluorouracil (FU). *Proc. Am. Assoc. Cancer Res.*, 38: 3188, 1997.
26. Chazal, M., Chéradame, S., Formento, J. L., Francoual, M., Formento, P., Etienne, M. C., François, E., Richelme, H., Mousseau, M., Letoublon, C., Pezet, D., Cur, H., Seitz, J. F., and Milano, G. Decreased folypolyglutamate synthetase activity in tumors resistant to fluorouracil-folinic acid treatment: clinical data. *Clin. Cancer Res.*, 3: 553–557, 1997.
27. Etienne, M. C., Guillot, T., and Milano, G. Critical factors for optimizing the 5-fluorouracil-folinic acid association in cancer chemotherapy. *Ann. Oncol.*, 7: 283–289, 1996.
28. Lu, K., Yin, M.-B., McGuire, J. J., Bonmassar, E., and Rustum, Y. M. Mechanisms of resistance to *N*-[5-[*N*-(3,4-dihydro-2-methyl-4-oxoquinazolin-6-ylmethyl)-*N*-methylamino]-2-thenoyl]-1-glutamic acid (ZD1694), a folate-based thymidylate synthase inhibitor, in the HCT-8 human ileocecal adenocarcinoma cell line. *Biochem. Pharmacol.*, 50: 391–398, 1995.
29. Drake, J. C., Allegra, C. J., Moran, R. G., and Johnston, P. G. Resistance to Tomudex (ZD1694): multifactorial in human breast and colon carcinoma cell lines. *Biochem. Pharmacol.*, 51: 1349–1355, 1996.
30. Jackman, A. L., Kelland, L. R., Kimbell, R., Brown, M., Gibson, W., Aherne, G. W., Hardcastle, A., and Boyle, F. T. Mechanisms of acquired resistance to the quinazoline thymidylate synthase inhibitor ZD1694 (Tomudex) in one mouse and three human cell lines. *Br. J. Cancer*, 71: 914–924, 1995.
31. Peters, G. J., Laurensse, E., Leyva, A., Lankelma, J., and Pinedo, H. M. Sensitivity of human, murine and rat cells to 5-fluorouracil and 5'-deoxy-5-fluorouridine in relation to drug-metabolizing enzymes. *Cancer Res.*, 46: 20–28, 1986.
32. Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72: 248–254, 1976.
33. Park, J.-G., Kramer, B. S., Steinberg, S. M., Carmichael, J., Collins, J. M., Minna, J. D., and Gazdar, A. F. Chemosensitivity testing of human colorectal carcinoma cell lines using a tetrazolium-based colorimetric assay. *Cancer Res.*, 47: 5875–5879, 1987.
34. Rutzy, L. P. The biology of human colon tumor cells in culture. *Adv. Cell Cult.*, 4: 47–83, 1985.

35. Skehan, P., Storeng, R., Scudiero, D., Monks, A., McMahon, J., Vistica, D., Warren, J. T., Bohesch, H., Kenney, S., and Boyd, M. R. New colorimetric cytotoxicity assay for anticancer-drug screening. *J. Natl. Cancer Inst.*, 82: 107–112, 1990.
36. Peters, G. J., Wets, M., Keeper, Y. P., Oskam, R., Van Ark-Otte, J., Noordhuis, P., Smid, K., and Pinedo, H. M. Transformation of mouse fibroblasts with the oncogenes *H-Ras* or *TRK* is associated with pronounced changes in drug sensitivity and metabolism. *Int. J. Cancer*, 3: 450–455, 1993.
37. Chou, T. C., and Talalay P. Quantitative analysis of dose-effect relationship: the combined effect of multiple drugs on enzyme inhibitors. *In: G. Weber (ed.), Advances in Enzyme Regulation*, pp. 27–55. New York: Pergamon Press, 1983.
38. Peters, G. J., Van Groeningen, C. J., Laurensse, E., and Pinedo, H. M. Thymidylate synthase from untreated human colorectal cancer and colonic mucosa: enzyme activity and inhibition by 5-fluoro-2'-deoxyuridine-5'-monophosphate. *Eur. J. Cancer*, 27: 263–267, 1991.
39. Peters, G. J., Laurensse, E., Leyva, A., and Pinedo, H. M. Purine nucleosides as cell-specific modulators of 5-fluorouracil metabolism and cytotoxicity. *Eur. J. Cancer Clin. Oncol.*, 23: 1869–1881, 1987.
40. Van Triest, B., Pinedo, H. M., Telleman, F., Van der Wilt, C. L., Jansen, G., and Peters, G. J. Cross-resistance to antifolates in multidrug resistant cell lines with P-glycoprotein or multidrug resistance protein expression. *Biochem. Pharmacol.*, 53: 1855–1866, 1997.
41. Van der Wilt, C. L., Smid, K., Aherne, G. W., Noordhuis, P., and Peters, G. J. Biochemical mechanisms of interferon modulation of 5-fluorouracil activity in colon cancer cells. *Eur. J. Cancer*, 33: 471–478, 1997.
42. Freemantle, S. J., Aherne, G. W., Hardcastle, A., Lunec, J., and Calvert, A. H. Increases in thymidylate synthase protein levels measured using newly developed antibodies. *Proc. Am. Assoc. Cancer Res.*, 32: 360, 1991.
43. Van der Wilt, C. L., Cloos, J., De Jong, M., Pinedo, H. M., and Peters, G. J. Screening of colon tumor cells and tissues for folylpolyglutamate synthetase activity. *Oncol. Res.*, 7: 317–321, 1995.
44. Braakhuis, B. J. M., Jansen, G., Noordhuis, P., Kegel, A., and Peters, G. J. Importance of pharmacodynamics in the *in vitro* antiproliferative activity of the antifolates methotrexate and 10-ethyl-10-deazaminopterin against human head and neck squamous cell carcinoma. *Biochem. Pharmacol.*, 46: 2155–2161, 1993.
45. Van der Laan, B. F. A. M., Jansen, G., Kathmann, G. A. M., Westerhof, G. R., Schornagel, J. H., and Hordijk, G. J. *In vitro* activity of novel antifolates against human squamous carcinoma cell lines of the head and neck with inherent resistance to methotrexate. *Int. J. Cancer*, 51: 909–914, 1992.
46. Jackman, A. L., Taylor, G. A., Gibson, W., Kimbell, R., Brown, M., Calvert, A. H., Judson, I. R., and Hughes, L. R. ICI D1694, a quinazoline antifolate thymidylate synthase inhibitor that is a potent inhibitor of L1210 tumor cell growth *in vitro* and *in vivo*: a new agent for clinical study. *Cancer Res.*, 51: 5579–5586, 1991.
47. Wang, F.-S., Aschele, C., Sobrero, A., Chang, Y.-M., and Bertino, J. R. Decreased folylpolyglutamate synthetase expression: a novel mechanism of fluorouracil resistance. *Cancer Res.*, 53: 3677–3680, 1993.
48. McGuire, J. J., Heitzman, K. J., Haile, W. H., Russell, C. A., McCloskey, D. E., and Piper, J. R. Cross-resistance studies of folylpolyglutamate synthetase-deficient, methotrexate-resistant CCRF-CEM human leukemia sublines. *Leuk. Res.*, 7: 1996–2003, 1993.
49. Jansen, G., Mauritz, R., Assaraf, Y. G., Sprecher, H., Drori, S., Kathmann, I., Westerhof, G. R., Priest, D. G., Bunni, M., Pinedo, H. M., Schornagel, J. H., and Peters, G. J. Regulation of carrier-mediated transport of folates and antifolates in methotrexate-sensitive and -resistant leukemia cells. *Adv. Enzyme Regul.*, 37: 59–76, 1997.
50. Jansen, G., Mauritz, R., Drori, S., Sprecher, H., Kathmann, I., Bunni, M., Priest, D. G., Noordhuis, P., Schornagel, J. H., Pinedo, H. M., Peters, G. J., and Assaraf, Y. G. A structurally altered human reduced folate carrier with increased folic acid transport mediates a novel mechanism of antifolate resistance. *J. Biol. Chem.*, 273: 30189–30198, 1998.
51. Tse, A., and Moran, R. G. Cellular folates prevent polyglutamylation of 5,10-dideazatetrahydrofolate. A novel mechanism of resistance to folate antimetabolites. *J. Biol. Chem.*, 273: 25944–25952, 1998.
52. Rees, C., Kimbell, R., Valenti, M., Brunton, L., Farrugia, D., and Jackman, A. L. Effects of leucovorin (LV) and folic acid (FA) on the cytotoxicity of the thymidylate synthase (TS) inhibitors, Tomudex (ZD1694) and ZD9331. *Proc. Am. Assoc. Cancer Res.*, 38: 3184, 1997.
53. Zervos, P. H., Allen, R. H., Thornton, D. E., and Thiem, P. A. Functional folate status as a prognostic indicator of toxicity in clinical trials of the multitargeted antifolate LY231514. *Eur. J. Cancer*, 33 (Suppl.): S18, 1997.
54. Smid, K., Van der Wilt, C. L., and Peters, G. J. Effects of novel antifolates on the binding of 5-fluoro-2'-deoxyuridine-5'-monophosphate (FdUMP) to bacterial and human TS. *Ann. Oncol.*, 7(suppl. 1): 310, 1996.
55. Stout, T. J., and Stroud, R. M. The complex of the anti-cancer therapeutic, BW1843U89, with thymidylate synthase at 2.0 Å resolution: implications for a new mode of inhibition. *Structure*, 4: 67–77, 1996.
56. Johnston, P. G., Mick, R., Recant, W., Behan, K. A., Dolan, M. E., Ratain, M. J., Beckmann, E., Weichselbaum, R. R., Allegra, C. J., and Vokes, E. E. Thymidylate synthase expression and response to neoadjuvant chemotherapy in patients with advanced head and neck cancer. *J. Natl. Cancer Inst.*, 89: 308–313, 1997.
57. Farrugia, D., Cunningham, D., Danenberg, P., Danenberg, K., Metzger, R., Mitchell, F., MacVicar, D., McCarthy, K., Aherne, G. W., Norman, A., and Jackman, A. L. A pharmacodynamic (PD) study of the thymidylate synthase (TS) inhibitor Tomudex in advanced colorectal cancer (CRC). *Proc. Am. Assoc. Cancer Res.*, 38: 4132, 1997.

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