0026-895X/91/020136-08$03.00/0

MOLECULAR PHARMACOLOGY, 39:136-143

Induction of Thymidylate Synthase Associated with Multidrug Resistance in Human Breast and Colon Cancer Cell Lines

EDWARD CHU, JAMES C. DRAKE, DAVID M. KOELLER, SYDELLE ZINN, CARLOS A. JAMIS-DOW, GRACE CHAO YEH, and CARMEN J. ALLEGRA

Medicine Branch, Division of Cancer Treatment, National Cancer Institute (E.C., J.C.D., S.Z., C.A.J.-D., G.C.Y., C.J.A.), and Cell Biology and Metabolism Branch, National Institute of Child Health and Human Development (D.M.K.), National Institutes of Health, Bethesda, Maryland 20892

Received September 12, 1990; Accepted November 19, 1990

SUMMARY

A series of Adriamycin-resistant human breast MCF-7 and human colon DLD-1 cancer cell lines were established by stepwise selection. The concentration of Adriamycin required to inhibit cell proliferation by 50% (IC_{50}) in the parent breast line (MCF-7), Adriamycin-resistant lines (MCF-Ad5 and MCF-Ad10), and a 5-fluorouracil (5-FU)-revertant line (MCF-R) was 0.005, 3.3, 6, and 4.9 μM, respectively. The Adriamycin IC_{50} value for the resistant colon line (DLD-Ad) was 8.2 μM, 68-fold higher than that for its parent line (DLD-1) (IC_{50} = 0.12 μM). The MCF-Ad5 and MCF-Ad10 cells were cross-resistant to 5-FU, with respective 5-FU IC_{50} values of 11.7 and 22.5 μM, or 7.3- and 14-fold less sensitive than their parent MCF-7 (IC_{50} = 1.6 μM) line. The MCF-R line completely reverted in sensitivity to 5-FU, with an IC_{50} of 1.7 μM. The resistant DLD-Ad line was 3.5-fold more resistant to 5-FU than was the parent DLD-1 line. Using both the 5-fluoro-2′-deoxyuridine-5′-monophosphate binding and catalytic assays for measurement of thymidylate synthase (TS) activity, there was significantly increased TS activity in the resistant MCF-Ad5 (2.4- and 2.5-fold), MCF-Ad10 (11.5- and 6.8-fold), and DLD-Ad (4.8- and 10.7-fold) lines, for binding and catalytic assays, respectively, compared with their parent MCF-7 and DLD-1 lines. The level of TS in cytosolic extracts, as determined by Western immunoblot analysis, was markedly increased for the resistant MCF-Ad5 (31-fold), MCF-Ad10 (46-fold), and DLD-Ad (52-fold) cells. Measurement of TS mRNA levels by Northern analysis revealed elevation of TS mRNA in the resistant MCF-AD5 (16.7-fold), MCF-Ad10 (31-fold), and DLD-Ad (55-fold) cells. Southern analysis showed that this increase in TS mRNA was not accompanied by any major rearrangements or amplification of the TS gene. Incorporation of 5-FU into the RNA and DNA of the resistant MCF-Ad10 cells was not significantly different, compared with that for parent MCF-7 cells. These studies suggest that exposure of human breast and human colon cancer cells to Adriamycin leads to overexpression of TS, with concomitant development of resistance to 5-FU.

The fluoropyrimidine 5-FU is an important antineoplastic agent and has displayed clinical efficacy in the treatment of solid tumors of the breast, head and neck, and gastrointestinal system (1–3). The established cytotoxic mechanisms of action of 5-FU have been previously well characterized and include 1) inhibition of TS by the metabolite FdUMP, with resultant inhibition of thymidylate formation and subsequent inhibition of DNA synthesis, 2) incorporation into RNA, and 3) incorporation into DNA (1–11).

One of the main obstacles to the clinical use of this drug has been the development of resistance to 5-FU. Various mechanisms of resistance to fluoropyrimidine chemotherapy have been well described (1, 3, 5, 12–20). They include the following: 1) relative deficiency of the intracellular folate substrate 5,10-methylene-H_4PteGlu, 2) enhanced activities of the catabolic enzymes acid and alkaline phosphatase, leading to a decreased intracellular accumulation of the active metabolites, 3) decreased levels of anabolic enzyme activities, with consequent decreased formation of the active metabolites, 4) decreased incorporation of 5-FU into RNA, and 5) changes in the target enzyme, TS, including altered affinity for FdUMP or increased intracellular enzyme activity. Recent studies by Scanlon and colleagues (21, 22) have shown cross-resistance to 5-FU in a human ovarian carcinoma A2780 cell line made resistant to cisplatin. Their analysis revealed that the resistance to 5-FU was due to a 3-fold increase in TS expression.

In the present study, we have established Adriamycin-resistant human breast MCF-7 and human colon DLD-1 carcinoma cell lines, by stepwise increases in the selective concentration of Adriamycin. These lines were markedly resistant to Adriamycin and, as well, were cross-resistant to 5-FU. These Adriamycin-resistant cell lines showed significant overexpression of TS, which appears to be associated with the development of resistance to 5-FU.

Materials and Methods

Chemicals. 5-FU, dUMP, dextran (clinical grade), bovine serum albumin fraction V, and acid-washed activated charcoal were purchased

ABBREVIATIONS: 5-FU, 5-fluorouracil; FdUMP, 5-fluoro-2′-deoxyuridine-5′-monophosphate; TS, thymidylate synthase; 5,10-methylene-H_4PteGlu, 5,10-methylenetetrahydrofolate; PBS, phosphate-buffered saline; SSC, sodium chloride/sodium citrate buffer; SDS, sodium dodecyl sulfate.

from Sigma Chemical Co. (St. Louis, MO). Adriamycin was supplied by Adria Laboratories (Columbus, OH). [6-^{3}H]FdUMP (18 Ci/mmol), [5-^{3}H]dUMP (20 Ci/mmol), [*methyl*-^{3}H]thymidine (60 Ci/mmol), and [6-^{3}H]5-FU (20 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). 5′-[α-^{32}P]dCTP (3000 Ci/mmol) was obtained from New England Nuclear (Boston, MA). All other chemicals were obtained from Sigma Chemical Co. or the National Institutes of Health supply room.

Cell culture. The origin of the human breast cancer MCF-7 cell line has been previously well described (23). The resistant sublines that were selected *in vitro* in a stepwise fashion for resistance to Adriamycin were maintained in medium containing Adriamycin at concentrations of 5 or 10 μM, for the MCF-Ad5 and MCF-Ad10 lines, respectively. The MCF-R line was derived by maintaining the MCF-Ad10 line in the absence of Adriamycin. The human colon cancer DLD-1 cell line was generously provided by Dr. A. Fojo (Medicine Branch, National Cancer Institute, Bethesda, MD). The resistant DLD-Ad line was selected *in vitro* for resistance to Adriamycin and was maintained in medium containing Adriamycin at a concentration of 5 μM. Cell lines were grown in 75-cm^2 plastic tissue culture flasks (Falcon Labware, Oxnard, CA), in growth medium containing RPMI 1640 with 10% dialyzed fetal bovine serum and 2 mM glutamine. Dialyzed fetal bovine serum was purchased from GIBCO (Grand Island, NY). All other medium components were obtained from Biofluids Co. (Rockville, MD). All of the resistant cell lines used in these experiments were maintained in the absence of Adriamycin for a minimum of 1 week before study.

Cell growth studies. Plastic 25-cm^2 tissue culture flasks (Falcon Labware) were seeded with 4.9-ml suspensions of 4–5 × 10^4 cells/ml MCF-7, MCF-Ad5, MCF-Ad10, MCF-R, DLD-1, and DLD-Ad cell lines and were incubated at 37°. After a 24-hr incubation, 0.1 ml of 5-FU or Adriamycin at various concentrations was added to each flask. Sterile water (0.1 ml) was added to control flasks. All experiments were carried out in duplicate. After a 72-hr incubation at 37°, cells were trypsinized and counted using a ZBI Coulter counter (Coulter Electronics Inc., Hialeah, FL). The concentration of drug that produced 50% inhibition of cell growth was determined from the plot of percentage of control growth (cell number) versus the logarithm of drug concentration.

In the thymidine-reversal growth experiments, the parent MCF-7 and resistant MCF-Ad10 lines were treated with thymidine, at a final concentration of 10^{-5} M, and 5-FU, at final concentrations of 10^{-6}, 3 × 10^{-6}, 10^{-5}, and 3 × 10^{-5} M. The cells were incubated at 37° for 72 hr, after which the cells were harvested and counted as described above.

The doubling times for the MCF-7, MCF-Ad5, MCF-Ad10, MCF-R, DLD-1, and DLD-Ad lines were 18, 22, 24, 20, 18, and 22 hr, respectively.

Measurement of DNA synthesis. Human breast MCF-7, MCF-Ad5, and MCF-Ad10 cancer cells, in the logarithmic phase of growth, were incubated with 1 μCi/ml [*methyl*-^{3}H]thymidine (specific activity, 60 Ci/mmol) for 30 min. The cells were denatured with 2 ml of 10% trichloroacetic acid and washed four times with ice-cold PBS, and the precipitated macromolecules were then solubilized with 1 ml of 1 M NaOH. A 0.5-ml aliquot of the solubilized preparation was assayed for radioactive counts, by being dissolved in 10 ml of liquid scintillation fluid (3a70B; Research Products Internatl. Corp., Mt. Prospect, IL) and counted in a liquid scintillation counter. The remainder of the preparation was assayed for protein content, according to the method of Bradford (24).

DNA synthesis in human breast MCF-7, MCF-Ad10, and MCF-R cancer cells was also determined by autoradiography, as described by Baserga and Malamud (25) and Whang-Peng *et al.* (26). In brief, cells in the logarithmic phase of growth were incubated with 1 μCi/ml [*methyl*-^{3}H]thymidine (specific activity, 60 Ci/mmol) for 2 hr at 37°. Cells were harvested in 10 ml of a hypotonic solution of 1% sodium citrate/0.075% potassium chloride. Autoradiography was performed on slides of acetic acid/ethanol-fixed cell suspensions, with Kodak NTB-2 autoradiography emulsion (Eastman Kodak, Rochester, NY). After an exposure time of 3 days at 4°, autoradiograms were developed in Kodak D-19b for 4 min at 15° and fixed in Kodak F5. The slides were then stained with Giemsa for cell counting. The thymidine labeling index was determined by scoring 1000 cells on slides prepared from each cell line and was defined as the percentage ratio between labeled and total tumor cells.

TS binding assay. Human breast MCF-7, MCF-Ad5, MCF-Ad10, and MCF-R and human colon DLD-1 and DLD-Ad cancer cells, in the logarithmic phase of growth, were washed three times with PBS, harvested, and resuspended in 0.1 M KH_2PO_4, pH 7.2. Cell lysis was accomplished by sonication using three 2–3-sec bursts. The extracts were centrifuged at 10,000 × *g* for 30 min, and the supernatants were immediately assayed. The reaction was carried out in a total volume of 200 μl, containing 75 μM 5,10-methylene-H_4PteGlu, 3 pmol of [6-^{3}H]FdUMP, 100 mM 2-mercaptoethanol, and 50 mM KH_2PO_4, pH 7.2. Samples were incubated at 37° for 30 min, following which 1 ml of a buffered charcoal slurry (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) was added. The mixtures were vortexed, allowed to stand at room temperature for 10 min, and then centrifuged at 10,000 × *g* for 30 min. An 800-μl sample of the supernatant was counted by liquid scintillation. The results were corrected for exchange of [^{3}H]FdUMP with bound nonradioactive FdUMP present in the cytosols. We have previously shown that 7% of FdUMP bound to TS is exchanged during 30 min under our assay conditions (27). Control experiments were performed, in which varying concentrations of Adriamycin (10^{-3} to 10^{-6} M) were added to the reaction mixtures. The inclusion of Adriamycin did not change the absolute levels of TS.

TS catalytic assay. Cell extracts from MCF-7, MCF-Ad5, MCF-Ad10, MCF-R, DLD-1, and DLD-Ad lines were obtained as described above. The assay was performed in a final volume of 200 μl, containing 10^{-5} M [5-^{3}H]dUMP, 100 mM 2-mercaptoethanol, 50 mM KH_2PO_4, pH 7.2, and 50 μl of cytosolic extract. Samples were incubated at 37° for 30 min. The reaction was stopped by addition of 100 μl of ice-cold 20% trichloroacetic acid. Unmetabolized [5-^{3}H]dUMP was removed by addition of 200 μl of an albumin-coated activated charcoal solution. The samples were vortexed and allowed to stand at room temperature for 10 min. The charcoal was sedimented by centrifugation at 10,000 × *g* for 30 min. A 250-μl sample of the supernatant was then assayed for [^{3}H]H_2O radioactivity by liquid scintillation counting.

FdUMP affinity determination (K_d). Cell extracts from MCF-7 and MCF-Ad10 were obtained as described above. The assay was performed in a final volume of 200 μl, containing 75 μM 5,10-methylene-H_4PteGlu, [6-^{3}H]FdUMP at concentrations ranging from 40 pM to 40 nM, 100 μM 2-mercaptoethanol, 50 mM K_2PO_4, and aliquots of cytosol (50 μl). The reaction mixtures were incubated for 30 min at 37°, enzyme-bound and free [6-^{3}H]FdUMP was determined, and affinity calculations were performed as previously described by Lockshin and Danenberg (28).

Incorporation of 5-FU into nucleic acids. Exponentially growing human breast MCF-7, MCF-Ad10, and MCF-R cancer cells were treated with [^{3}H]5-FU (1 μM; final specific activity, 40 μCi/mmol) for 4 hr at 37°. The cells were then washed three times with ice-cold PBS and fractionated for RNA and DNA fractions, as outlined previously by Chu and Fischer (29, 30) and Harkrader *et al.* (31). Cellular RNA was hydrolyzed in 0.2 M NaOH, and DNA was hydrolyzed in 1 M perchloric acid. Then, 200-μl samples of the hydrolysates were added to scintillation vials containing 10 ml of 3a70B counting cocktail, and tritium radioactivity was measured in a Packard Tricarb liquid scintillation counter.

Isolation of total RNA. Human breast and human colon cancer cells were harvested from 150-cm^2 tissue culture flasks with a rubber policeman and washed three times with ice-cold PBS, and their total RNA was isolated according to the method of Chomczynski and Sacchi (32). After extraction, the RNA was stored at −80° for use in Northern blot analysis.

RNA blot hybridization (Northern) analysis. For Northern

transfer analysis, 30 μg/sample of total cellular RNA were denatured, fractionated on a 1% formaldehyde-agarose gel, and transferred to a Nytran filter membrane (Schleicher and Schuell, Keene, NH), by a modification of the method of Ayusawa *et al.* (33). The membrane was baked for 2 hr at 80° in a vacuum oven. Prehybridization of the membrane for 2 hr at 42° in 50% formamide, 5× SSC, 5× Denhardt's solution, 20 mM Na_2HPO_4/NaH_2PO_4, pH 7.4, 200 μg/ml salmon sperm DNA, 0.1% SDS, was then followed by hybridization for 24 hr in the same solution with 1×10^6 cpm/ml ^{32}P-labeled nick-translated TS cDNA probe. The cDNA for human TS was a generous gift from Dr. T. Seno (Saitama Cancer Center Research Institute, Saitama-ken, Japan). The hybridized filter was initially washed twice with 2× SSC at room temperature, followed by a 1-hr wash at 65° with 0.2× SSC, 0.1% SDS. Autoradiography was performed using Kodak XAR-5 film exposed for 2–4 days at −70°.

Western immunoblot analysis. Human breast and colon cancer cells were harvested in PBS and centrifuged at 1000 × *g* for 10 min at 4°. Cell extracts were then prepared as described above. Protein concentrations were determined by the Bio-Rad protein assay (24), and equivalent amounts of protein from each sample were resolved by SDS-polyacrylamide gel electrophoresis, using 15% acrylamide, according to the method of Laemmli (34). The gels were then electroblotted onto nitrocellulose membranes. Antibody staining was performed with the use of a TS polyclonal primary antiserum (1/10000 dilution) and a horseradish peroxidase-conjugated secondary antibody (1/10000 dilution) (Bio-Rad Laboratories, Richmond, CA). The TS polyclonal antibody was raised against HeLa TS in rabbits and was a generous gift from Dr. B. Yates and Dr. I. K. Dev (Wellcome Research Laboratories, Burroughs Wellcome Co., Research Triangle Park, NC).

Extraction and analysis of DNA. Exponentially growing cells were washed twice with ice-cold PBS, scraped with a rubber policeman, and treated with a nucleic acid extraction buffer, containing 1% SDS, 0.4 M NaCl, 10 mM EDTA, 10 mM Tris · HCl, pH 7.6, and proteinase K (150 μg/ml), at 37° for an overnight incubation. After phenol/chloroform extraction and ethanol precipitation, nucleic acids were then treated with RNase (100 μg/ml) at 37° for 1 hr. A repeat phenol/chloroform extraction and ethanol precipitation were performed, after which the DNA was dissolved in 2 mM Tris · HCl, pH 7.4, 1 mM EDTA. Southern analysis was done by digestion of 15 μg of the isolated genomic DNA with the appropriate restriction endonucleases, fractionation of the DNA fragments on 0.8% agarose gels, and transfer to a Nytran filter membrane (Schleicher and Schuell). The membrane was hybridized to a ^{32}P-radiolabeled human TS cDNA insert probe and then processed exactly as described above for the Northern blot analysis.

Protein determination. Protein concentration was determined according to the Bio-Rad protein assay, based on the Bradford dye-binding procedure (24).

Results

***In vitro* cytotoxicity studies.** The MCF-Ad5 and MCF-Ad10 human breast cancer cell lines were selected sequentially by exposure of the parent MCF-7 line to stepwise increases in Adriamycin concentration in the selection medium. Once the degree of resistance remained stable at each step (the IC_{50} was the same in two successive determinations), the cells were then maintained in 5 or 10 μM Adriamycin, respectively. As shown in Table 1, the growth inhibition assay revealed that the Adriamycin IC_{50} values for the MCF-Ad5 and MCF-Ad10 lines were 4.3 and 9 μM, respectively. These lines were 860- and 1800-fold more resistant to Adriamycin than was the parent MCF-7 line (IC_{50} = 5 nM). The MCF-R line remained resistant to Adriamycin (IC_{50} = 4.9 μM), despite being in drug-free medium for up to 40 weeks. The sensitivity of these four cell lines to 5-FU was then determined. The 5-FU IC_{50} values for the MCF-Ad5 and MCF-Ad10 lines were markedly increased, 11.7 and 22.7 μM, respectively, whereas the parent MCF-7 and the MCF-R lines had respective IC_{50} values of 1.6 and 1.7 μM. In all determinations of Adriamycin and 5-FU cytoxicity against the resistant cell lines, at least four concentrations of each drug were tested against parent MCF-7 cells, to ensure that the cytotoxic activity of the drug was consistent from one experiment to the next.

TABLE 1

Growth-inhibitory effect (IC_{50}) of 5-FU and Adriamycin against human breast cancer (MCF-7) cells and human colon cancer (DLD-1) cells in culture

Human breast and colon cancer cells in the exponential phase of growth were incubated with Adriamycin (10^{-4} to 10^{-10} M) and 5-FU (10^{-4} to 10^{-7} M) at 37° for 72 hr. The IC_{50} values (concentrations producing 50% growth inhibition) were determined as described in Materials and Methods. The MCF-7 line represents the parent breast cancer line, and MCF-R is the revertant line of MCF-Ad10 that was maintained in the absence of Adriamycin for more than 40 weeks. The resistant MCF-Ad5 and MCF-Ad10 lines were maintained in Adriamycin concentrations of 5 and 10 μM, respectively. The DLD-1 line represents the parent colon cancer line, and DLD-Ad was maintained in 5 μM Adriamycin. Results shown represent mean ± standard error from at least five separate experiments, each done in duplicate.

Cell line	IC_{50}	
	Adriamycin	5-FU
	μM	
MCF-7	0.005 ± 0.001	1.6 ± 0.5
MCF-Ad5	4.30 ± 1.20	11.7 ± 2.8
MCF-Ad10	9.00 ± 1.20	22.5 ± 2.6
MCF-R	4.90 ± 1.00	1.7 ± 0.7
DLD-1	0.10 ± 0.04	8.2 ± 0.2
DLD-Ad	7.20 ± 1.40	29.0 ± 3.5

The stability of the resistance phenotype in the absence of Adriamycin was examined in the most resistant line, MCF-Ad10 (Fig. 1A). When this line was passaged without Adriamycin for 2, 4, 8, 12, and 40 weeks, the Adriamycin IC_{50} remained unchanged. In contrast, the resistance of this cell line to 5-FU required the continued presence of Adriamycin in the growth medium. The 5-FU IC_{50} decreased to 6 μM when these cells were drug-free for 2 weeks, and by 4 weeks these MCF-R cells had reverted to a sensitivity to 5-FU that was indistinguishable from that of the parent MCF-7 cells, with an IC_{50} of 1.7 μM. When the MCF-R cells were then regrown and maintained in 10 μM Adriamycin, they quickly regained resistance to 5-FU. These cells were 3-fold resistant to 5-FU 2 weeks after being placed in drug-containing medium (Fig. 1B). By 10 weeks, these MCF-R cells were maximally resistant to 5-FU, with an IC_{50} of 25 μM (Fig. 1B).

Similar growth studies were performed on the human colon carcinoma line DLD-1 and its corresponding Adriamycin-resistant line, DLD-Ad. The DLD-Ad line was 68-fold more resistant to Adriamycin than its parent DLD-1 line. As seen in Table 1, when these lines were tested for sensitivity to 5-FU, the DLD-Ad line was 3.5-fold more resistant to 5-FU than was the DLD-1 parent line. When the DLD-Ad line was passaged without Adriamycin for more than 4 weeks, its sensitivity to 5-FU was identical to that of the parent cell line, with an IC_{50} of 8 μM (data not shown).

In the thymidine-reversal growth studies, the presence of thymidine completely protected against the growth-inhibitory activity of 5-FU, in both the parent and resistant MCF-Ad10 cells (data not shown). These results suggest that inhibition of TS or some other DNA-mediated process may be an important determinant of 5-FU cytotoxicity in these two cell lines.

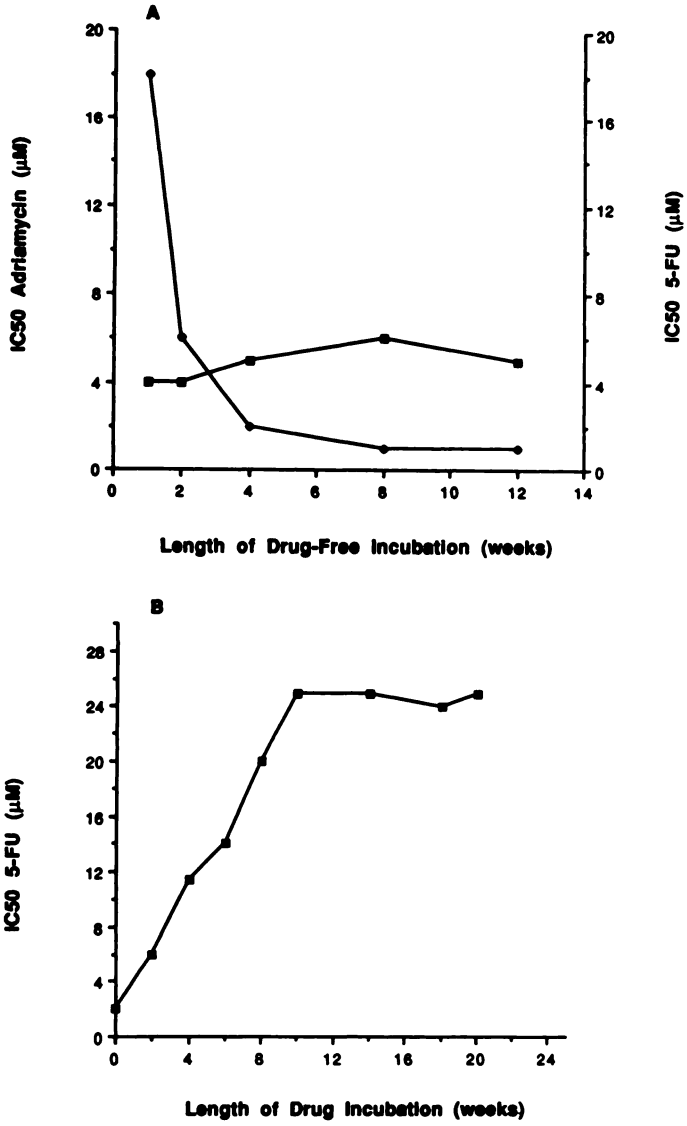

Fig. 1. A, Effects of maintenance in Adriamycin-free medium on resistance of MCF-Ad10 to 5-FU and Adriamycin. The MCF-Ad10 line was incubated in the absence of Adriamycin and, at the intervals indicated, was assayed for sensitivity to 5-FU (♦) and Adriamycin (□), as described in Materials and Methods. IC_{50} values were determined using at least five drug concentrations, with each concentration assayed in duplicate. B, The MCF-R line was incubated in the presence of 10 μM Adriamycin and, at the intervals indicated, was assayed for sensitivity to 5-FU, as described in Materials and Methods.

Measurement of DNA synthesis. Incorporation of [^{3}H] thymidine into DNA was the same for parent MCF-7 (2.1 nmol/mg of DNA) and revertant MCF-R (2.3 ± 0.2 nmol/mg of DNA) cells. There was a slight increase in thymidine incorporation into the DNA of the resistant MCF-Ad10 (3.0 ± 0.2 nmol/mg of DNA) cells.

When light microscopic autoradiography with [^{3}H]thymidine was performed to more accurately determine the proliferative activity of each of these cell lines, the thymidine labeling index for the parent MCF-7 (47 ± 2%), resistant MCF-Ad10 (41 ± 4%), and revertant MCF-R (46 ± 3%) cell lines was equivalent.

TS as a determinant of resistance. Increased expression of the critical target enzyme, TS, is a principal mechanism of resistance to 5-FU. TS activity, as measured by the FdUMP binding assay, was 2.4- and 11.5-fold increased in the resistant MCF-Ad5 and MCF-Ad10 lines, respectively, when compared with the TS activity in the parent MCF-7 line (Table 2). These values were significantly different for both the MCF-Ad5 (p_2 = 0.04) and the MCF-Ad10 (p_2 = 0.0001) lines, when compared with the parent cell line. The revertant MCF-R and parent MCF-7 lines had equivalent TS activity. Measurement of FdUMP binding activity in the human colon DLD-Ad line revealed a 4.8-fold increase in TS level, compared with its parent DLD-1 line (p_2 = 0.0001). We found that the relative differences in TS levels among parent and Adriamycin-resistant human breast and colon cancer cells were equivalent when standardized either to cytosolic protein concentration or to cell count (data not shown).

Using the FdUMP binding assay, TS activity in sensitive MCF-7 and resistant MCF-Ad10 cells was measured following 24-hr exposure to concentrations of either 1 μM Adriamycin or 1 μM 5-FU. There were no measurable differences in TS activity after exposure to either drug, when TS activities measured before and immediately after acute drug exposures were compared (data not shown).

TS activity, as measured by the catalytic assay, was 2.7- and 6.8-fold increased in the MCF-Ad5 and MCF-Ad10 lines, respectively, when compared with the parent MCF-7 line (Table 2). The MCF-R line had slightly lower TS catalytic activity than the parent MCF-7 line. The resistant DLD-Ad line had 10.7-fold increased TS catalytic activity, compared with its parent DLD-1 line.

In all of these experiments measuring TS activity, the Adriamycin-resistant cell lines were maintained in medium without Adriamycin for at least 1 week. We also measured TS activity in the resistant MCF-Ad5 and MCF-Ad10 lines when these cell lines were maintained in Adriamycin-containing media. Under these conditions, as determined by the FdUMP binding assay, TS activity was equivalent to that obtained when these cells were maintained in the absence of Adriamycin (data not shown).

The tightness of binding of FdUMP to TS extracted from both sensitive MCF-7 and 5-FU-resistant MCF-Ad10 breast cancer cells was determined by measuring the respective dissociation constants, K_d, of their TS-FdUMP-5,10-methylene-H_4PteGlu complexes. There was no difference in TS binding affinity, inasmuch as the K_d values for FdUMP binding to TS of the sensitive MCF-7 and resistant MCF-Ad cells were 0.67 and 0.61 nM, respectively (data not shown).

Western immunoblot analysis. Cell extracts were ana-

TABLE 2

TS activity in human breast and human colon cancer cells

Human breast and human colon cancer cells in the logarithmic phase of growth were harvested, and cytosolic extracts served as the source of enzyme. TS activity was determined using the FdUMP binding and catalytic assays, as described in Materials and Methods. Results shown are mean ± standard error of at least four separate experiments, each done in duplicate. The MCF-Ad5 (p_2 = 0.04), MCF-Ad10 (p_2 = 0.0001), and DLD-Ad (p_2 = 0.0001) lines were associated with a significantly increased level of TS, as determined by the FdUMP binding assay. Statistical analysis was performed using a two-tailed Student's *t* test.

Cell line	Binding assay	Catalytic assay
	pmol/mg of protein	*pmol/min/mg of protein*
MCF-7	0.16 ± 0.04	4.0 ± 0.2
MCF-Ad5	0.38 ± 0.09	11.0 ± 3.0
MCF-Ad10	1.84 ± 0.27	27.0 ± 5.0
MCF-R	0.11 ± 0.05	2.4 ± 0.2
DLD-1	1.45 ± 0.07	3.7 ± 0.14
DLD-Ad	7.00 ± 0.30	39.6 ± 2.10

lyzed by Western blot, to determine whether the differences in TS activities corresponded to changes in the amount of immunoreactive enzyme, as illustrated in Fig. 2. Equivalent amounts of total cytosolic protein (200 μg) were loaded onto each lane. In addition, 1 ng of purified human recombinant TS (a generous gift from Dr. Daniel Santi, Department of Biochemistry and Biophysics, University of California, San Francisco) was loaded onto one lane, to verify the migration position of TS. The primary TS antiserum employed in these experiments was polyclonal in nature and, thus, stained a number of bands. It is known, however, that the molecular weight of the denatured monomeric form of TS is 35,000, which was confirmed by the position of staining with purified human recombinant TS. As seen in Fig. 2, *lanes B* and *E*, representing MCF-7 and MCF-R, reveal no staining for TS. However, Fig. 2, *lanes C* and *D*, representing MCF-Ad5 and MCF-Ad10, reveal significant staining for TS. Negligible amounts of TS were noted in the parental human colon cancer DLD-1 line (Fig. 2, *lane F*), whereas there is significant TS in Fig. 2, *lane G*, representing DLD-Ad. Quantitation of the band densities by densitometric scanning of photograph negatives revealed 31- and 46-fold increased expression of TS, respectively in the MCF-Ad5 and MCF-Ad10 lines. Similar analysis of the human colon cancer lines showed a 52-fold increased expression of TS in the resistant DLD-Ad line.

RNA blot hybridization and Southern blot analysis. Analysis of TS mRNA levels by Northern blot hybridization (Fig. 3) revealed a single 1.6-kilobase mRNA species that hybridized with the radiolabeled human TS cDNA. There were 16.7- and 31-fold elevated TS mRNA levels, respectively, in the MCF-Ad5 (Fig. 3, *lane D*) and MCF-Ad10 (Fig. 3, *lane C*) lines, compared with the parent MCF line (Fig. 3, *lane A*). The revertant MCF-R line (Fig. 3, *lane B*) had the same level of TS mRNA as the parent MCF-7 line. There were 55-fold increased TS mRNA levels in the DLD-Ad cells (Fig. 3, *lane F*), compared with parental DLD-1 cells (Fig. 3, *lane E*). The mRNA from each of these lines appeared intact and equally loaded and transferred, based on ethidium bromide staining of the gel and on hybridization analysis of the blot with a radiolabeled human β-actin probe (Fig. 3).

In order to determine the potential roles of structural gene

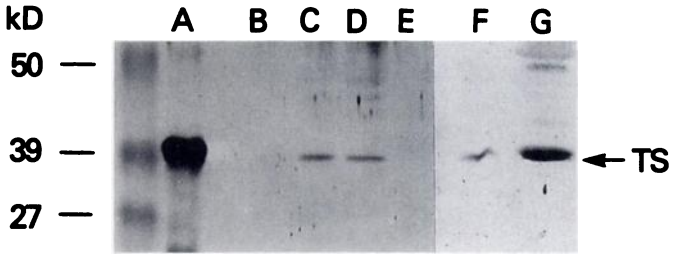

Fig. 2. Western immunoblot analysis of TS from parent and Adriamycin-resistant MCF-7 and DLD-1 cells. Cytosolic extracts from cells in the exponential phase of growth were prepared as described in Materials and Methods. Proteins (200 μg) were resolved on a SDS-polyacrylamide gel. Staining of the Western blot was performed using polyclonal TS primary antiserum (1/10000 dilution) and horseradish peroxidase-conjugated secondary antibody (1/10000 dilution). Human recombinant TS protein (1 ng) was applied to the gel to verify the position of TS staining (*lane A*). Proteins were from parent MCF-7 (*lane B*), resistant MCF-Ad10 (*lane C*) and MCF-Ad5 (*lane D*), revertant MCF-R (*lane E*), parent DLD-1 (*lane F*), and resistant DLD-Ad (*lane G*) cells. Quantitation of signal densities was performed by densitometric scanning (Beckman DU-65 spectrophotometer).

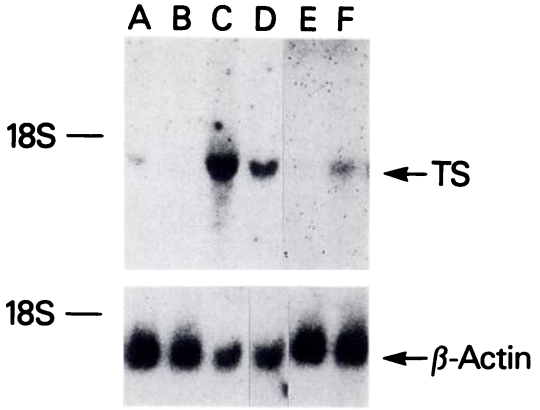

Fig. 3. Northern blot analysis of TS mRNA in parent and Adriamycin-resistant MCF-7 and DLD-1 cells. Total RNA (30 μg) from each cell line was fractionated on a 1% formaldehyde-agarose gel, transferred to a Nytran membrane, hybridized with ^{32}P-radiolabeled TS cDNA insert, and autoradiographed, as described in Materials and Methods. TS mRNA is 1.6 kilobases. The filters were then stripped of the TS probe and rehybridized with a human β-actin probe, to control for loading and integrity of mRNA. RNAs were from parent DLD-1 (*lane E*), resistant DLD-Ad (*lane F*), resistant MCF-Ad10 (*lane C*) and MCF-Ad5 (*lane D*), revertant MCF-R (*lane B*), and parent MCF-7 (*lane A*) cells. Quantitation of signal densities was performed by densitometric scanning (Beckman DU-65 spectrophotometer).

TABLE 3

Incorporation of 5-FU into RNA and DNA of human breast cancer cells

Human breast cancer cells in the exponential phase of growth were incubated with [6-^{3}H]5-FU (1 μM; final specific activity, 40 μCi/mmol) at 37° for 4-hr. Cells were extracted and fractionated for RNA and DNA, as described in Materials and Methods. Results shown are mean ± standard error of at least four separate experiments.

Cell line	5-FU incorporation	
	RNA	DNA
	pmol/mg of RNA	*pmol/mg of DNA*
MCF-7	30 ± 1.0	0.46 ± 0.08
MCF-Ad10	15 ± 6.8	0.80 ± 0.10
MCF-R	19 ± 5.1	0.42 ± 0.05

rearrangement and amplification in TS mRNA elevation, analysis of TS genes in parent and resistant MCF-7 and DLD-1 lines by Southern blotting of genomic DNA was performed. The genomic DNAs were digested with restriction endonucleases *Hind*III and *Eco*RI, fractionated by agarose gel electrophoresis, and transferred onto a Nytran membrane. The blots were then incubated with a radiolabeled human TS cDNA insert. All the DNA fragments generated by restriction digestion were of the same size and intensity in the parent MCF-7 and resistant DLD-1 lines, suggesting no significant rearrangements of gene structure or amplification of TS-specific DNA (data not shown).

Incorporation into nucleic acids. Incorporation into both RNA and DNA has been correlated with 5-FU cytotoxicity in a number of *in vitro* and *in vivo* systems. Consequently, we determined the amount of 5-FU incorporation into both of these nucleic acids. As shown in Table 3, the level of 5-FU incorporation into the RNA of the most resistant line, MCF-Ad10, was 50% of that found in the parent MCF-7 line. The extent of 5-FU incorporation into the RNA of the revertant MCF-R line, however, was 60% of that in the parent cell line and not significantly different from that obtained in the MCF-Ad10 line, 19 pmol/mg versus 15 pmol/mg, respectively.

The levels of 5-FU incorporation into the DNA of MCF-7 and MCF-R cells were equivalent, whereas the resistant MCF-Ad10 line had 2-fold greater levels of incorporated fluorinated pyrimidines in its DNA (Table 3).

Discussion

The results of the present study demonstrate that human breast MCF-7 and human colon DLD-1 cancer cells selected for resistance to Adriamycin develop cross-resistance to 5-FU. Although the degree of cross-resistance to 5-FU is substantially less than the degree of resistance to the selecting agent, Adriamycin, this observation is consistent with that reported by others, in that the greatest resistance occurs against the selecting agent and cross-resistance to other drugs is less (35–37). Comparison of the stability of the resistance phenotype in the breast cancer cell lines, however, reveals a significant difference between these two agents. When resistant MCF-Ad10 cells were maintained in Adriamycin-free medium for up to 8 months, there was no change in Adriamycin IC_{50} values. In contrast, there was a dramatic decrease in the relative 5-FU resistance when these cells were removed from Adriamycin-containing medium for a 4- to 6-week period.

Previous studies have defined overexpression of multidrug-resistance gene (*mdr-1*) mRNA and its protein product, P-glycoprotein (38), as well as overexpression of glutathione-*S*-transferase (39), as playing potentially important roles in the development of resistance to Adriamycin in these resistant MCF-7 breast cancer cells. Our studies demonstrate that the process of Adriamycin selection resulted in significantly increased expression of TS. This result has been confirmed using biochemical methods, including FdUMP binding and TS catalytic assays, as well as Western immunoblot analysis. A resistant human colon cancer line, DLD-Ad, was established by exposure of the parent DLD-1 line to Adriamycin-containing medium. As noted in the resistant breast cancer cells, there was a similar increase in TS protein expression in the resistant DLD-Ad line. Moreover, this increase in TS level was associated with development of cross-resistance to 5-FU. The association between intracellular levels of TS protein and 5-FU sensitivity is further strengthened by the characteristics of the revertant MCF-R line, in which the cells have regained sensitivity to 5-FU and have a similar TS level and catalytic activity as the parent MCF-7 line. Thus, taken together, these findings indicate that the specific mechanisms of resistance to Adriamycin and to 5-FU in these cell lines are different. However, the continued selective pressure of Adriamycin within the growth medium appears to be a critical factor in establishing and maintaining the enhanced intracellular expression of TS protein and subsequent cross-resistance to 5-FU.

A number of studies have previously demonstrated that TS is expressed primarily in the S phase of the cell cycle (40–45). Given these earlier studies, we determined whether Adriamycin might be affecting the proportion of cells in the S phase, for the Adriamycin-resistant cells. We used two different techniques; the first was a thymidine incorporation study and the second was an autoradiographic study of thymidine labeling index, to assess the proliferative rate of these cells. Our results indicate that the increased expression of TS protein in the Adriamycin-resistant MCF-Ad10 cells is not due to an enhanced rate of DNA synthesis in these cells, because the levels of thymidine incorporation and the thymidine labeling indices of the parent MCF-7, resistant MCF-Ad10, and revertant MCF-R cells were equivalent.

Although the direction of change in TS expression, as determined by the biochemical TS binding assay and the Western immunoblot system, was equivalent for parent, resistant, and revertant human breast and colon cancer cell lines, the relative changes in TS levels were different. In all the cell lines exposed to Adriamycin, the Western immunoblot analysis revealed a more dramatic increase in TS than did the TS binding assay. Our Scatchard analysis of the TS protein from parent MCF-7 and resistant MCF-Ad10 cells revealed no alterations in affinity of TS for FdUMP, suggesting that the TS protein in these two lines was qualitatively the same. Although differences in sensitivity between the two assays may account for some of this observed discrepancy, it is conceivable that some of the increased amount of immunoreactive TS protein is biologically nonfunctional.

The increase in immunoreactive TS in the Adriamycin-resistant breast and colon cancer cells was associated with changes in the steady state levels of TS mRNA. As shown in Fig. 3, Northern blot hybridization revealed significant elevations in TS mRNA content in the resistant MCF-Ad5, MCF-Ad10, and DLD-Ad cells, compared with revertant MCF-R and parent MCF-7 and DLD-1 cells. This increase in TS mRNA, however, was not the result of amplification or structural gene rearrangements in the TS gene, because Southern analysis did not suggest any qualitative or quantitative differences between resistant and parent human breast and colon lines. Thus, the increase in overall TS activity and the increase in immunoreactive cytosolic TS in the resistant breast and colon cancer cells suggest either a transcriptional or a posttranscriptional regulatory event. Scanlon and co-workers (21, 22) previously reported that human ovarian cancer A2780 cells made resistant to cisplatin were cross-resistant to 5-FU. Furthermore, their study provided evidence suggesting that the expression of TS protein in this cisplatin-resistant line was regulated at a transcriptional level. A number of other studies have also shown that expression of the TS gene is controlled at both the transcriptional and posttranscriptional levels (42–47).

We have examined other potential mechanisms that might contribute to the development of the 5-FU resistance phenotype in the Adriamycin-resistant breast cancer cells. Specifically, we have compared the level of 5-FU incorporation into the RNA and DNA of resistant MCF-Ad10 and parent MCF-7 cells. As seen in Table 3, there was 2-fold less incorporation of 5-FU into RNA of the resistant line, compared with its parent line. However, the revertant MCF-R line had the same level of incorporated 5-FU as the resistant line from which it was derived. Thus, this result, along with the fact that the revertant MCF-R line had completely regained sensitivity to 5-FU, suggests that the decreased level of 5-FU incorporation into RNA is not a major determinant of 5-FU resistance in these cell lines. Moreover, our growth studies show that thymidine completely protects against the growth-inhibitory effects of 5-FU in the resistant MCF-Ad10 line, consistent with the reasoning that RNA-mediated effects may not be an important determinant of 5-FU cytotoxicity in this cell line.

The Adriamycin-resistant MCF-7 breast cells have been previously well characterized, in terms of the underlying mechanisms responsible for their Adriamycin resistance (38, 39). Overexpression of the *mdr-1* gene and its protein product P-

glycoprotein, with a resultant decrease in intracellular drug accumulation, appears to be an important process associated with Adriamycin resistance (35, 36, 48–53). Other studies have shown that P-glycoprotein expression is increased in normal and malignant tissues of the liver, kidney, and intestine, perhaps in response to constant exposure to naturally occurring toxins (54).

A number of recent studies have shown that cross-resistance can develop between Adriamycin and the antifolates methotrexate and trimetrexate. Using the Chinese hamster cell model, Rice *et al.* (55) have determined that the observed frequency of simultaneous acquisition of resistance to methotrexate and Adriamycin is 10–100 times higher than that predicted from the independent frequencies of each selected alone. Recent studies by Assaraf *et al.* (56) and Arkin *et al.* (37) provide evidence that cross-resistance to the antifolate trimetrexate is associated with the multidrug-resistance phenotype. Our observation that treatment with Adriamycin results in cross-resistance to the antimetabolite 5-FU suggests that malignant cells have a variety of intrinsic adaptive mechanisms to circumvent the effects of a host of cytotoxic stresses. Induction of TS in response to continuous exposure to Adriamycin may represent a situation similar to induction of P-glycoprotein in response to natural toxins and to the natural products. Because normal and malignant cells of the intestine are constantly exposed to natural toxins, it is conceivable that TS expression in these cells is maintained at an increased level. Thus, this TS-dependent resistance may, in part, explain the significant *de novo* resistance of gastrointestinal malignancies to fluoropyrimidine therapy.

In summary, human breast MCF-7 and human colon DLD-1 cancer cell lines that are resistant to Adriamycin develop cross-resistance to 5-FU. Our analysis suggests that this resistance to 5-FU is due to an enhanced expression of TS protein. Additional studies are needed to more specifically define the underlying molecular mechanisms of the induction of TS by Adriamycin. However, this observation that exposure to Adriamycin can lead to resistance to 5-FU may have clinical implications, because both of these agents are commonly used in the treatment of human malignancies.

Acknowledgments

The authors would like to thank Dr. Bruce Chabner and. Dr. Jacqueline Whang-Peng for their insightful and helpful discussions and Kathy Moore for her editorial assistance in the preparation of this manuscript.

References

1. Pinedo, H. M., and G. F. J. Peters. Fluorouracil: biochemistry and pharmacology. *J. Clin. Oncol.* **6**:1653–1664 (1988).
2. Heidelberger, C. Fluorinated pyrimidines and their nucleosides, in *Antineoplastic and Immunosuppressive Agents* (A. Sartorelli and D. Johns, eds). Springer-Verlag, New York, 193–231 (1975).
3. Heidelberger, C., P. V. Danenberg, and R. G. Moran. Fluorinated pyrimidines and their nucleosides. *Adv. Enzymol. Relat. Areas Mol. Biol.* **54**:57–119 (1989).
4. Santi, D. V., C. S. McHenry, and H. Sommer. Mechanism of interaction of thymidine synthetase with 5-fluorodeoxyuridylate. *Biochemistry* **13**:471–481 (1974).
5. Ardalan, B., D. A. Cooney, H. N. Jayaram, C. K. Carrico, R. I. Glazar, J. Macdonald, and P. S. Schein. Mechanisms of sensitivity and resistance of murine tumors to 5-fluorouracil. *Cancer Res.* **40**:1431–1437 (1980).
6. Spiegelman, S., R. Sawyer, R. Nayak, E. Ritzi, R. Stolfi, and D. Martin. Improving the antitumor activity of 5-fluorouracil by increasing its incorporation into RNA via metabolic modulation. *Proc. Natl. Acad. Sci. USA* **77**:4966–1970 (1980).
7. Wilkinson, D. S., and J. Crumley. The mechanism of 5-fluorouridine toxicity in Novikoff hepatoma cells. *Cancer Res.* **36**:4032–4038 (1976).
8. Glazar, R. I., and A. L. Peale. The effect of 5-fluorouracil on the synthesis of nuclear RNA in L1210 cells *in vitro*. *Mol. Pharmacol.* **16**:270–277 (1979).
9. Kufe, D. W., P. P. Major, E. M. Egan, and E. Loh. 5-Fluoro-2′-deoxyuridine incorporation in L1210 DNA. *J. Biol. Chem.* **256**:8885–8888 (1981).
10. Major, P. P., E. Egan, D. Herrick, and D. W. Kufe. 5-Fluorouracil incorporation in DNA of human breast carcinoma cells. *Cancer Res.* **42**:3005–3009 (1982).
11. Cheng, Y.-C., and K. Nakayama. Effects of 5-fluoro-2′-deoxyuridine on DNA metabolism in HeLa cells. *Mol. Pharmacol.* **23**:171–174 (1983).
12. Houghton, J. A., K. D. Weiss, L. G. Williams, P. M. Torrance, and P. J. Houghton. Relationship between 5-fluoro-2′-deoxyuridylate, 2′-deoxyuridylate and thymidylate synthase activity subsequent to 5-fluorouracil administration, in xenografts of human colon adenocarcinomas. *Biochem. Pharmacol.* **35**:1351–1358 (1986).
13. Houghton, J. A., S. J. Maroda, J. O. Phillips, and P. J. Houghton. Biochemical determinants of responsiveness to 5-fluorouracil and its derivatives in xenografts of human colorectal adenocarcinomas in mice. *Cancer Res.* **41**:144–149 (1981).
14. Yin, M. B., S. F. Zakrzewski, and M. T. Hakala. Relationship of cellular folate cofactor pools to the activity of 5-fluorouracil. *Mol. Pharmacol.* **23**:190–197 (1983).
15. Mulkins, M. A., and C. Heidelberger. Biochemical characterization of fluoropyrimidine-resistant murine leukemic cell lines. *Cancer Res.* **42**:965–973 (1982).
16. Fernandes, D. J., and S. K. Crawford. Resistance of CCRF-CEM cloned sublines to 5-fluorodeoxyuridine associated with enhanced phosphatase activities. *Biochem. Pharmacol.* **34**:125–132 (1985).
17. Berger, S. H., C.-H. Jenh, L. F. Johnson, and F. Berger. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol. Pharmacol.* **28**:461–467 (1985).
18. Clark, J. L., S. H. Berger, A. Mittelman, and F. Berger. Thymidylate synthase gene amplification in a colon tumor resistant to fluoropyrimidine chemotherapy. *Cancer Treat. Rep.* **71**:261–265 (1987).
19. Berger, S. H., K. W. Barbour, and F. Berger. A naturally occurring variation in thymidylate synthase structure is associated with a reduced response to 5-fluoro-2′-deoxyuridine in a human colon tumor cell line. *Mol. Pharmacol.* **34**:480–484 (1988).
20. Kessel, D., T. C. Hall, and I. Wodinsky. Nucleotide formation as a determinant of 5-fluorouracil response in mouse leukemia. *Science (Washington D. C.)* **154**:911–913 (1966).
21. Scanlon, K. J., and M. Kashani-Sabet. Elevated expression of thymidylate synthase cycle genes in cisplatin-resistant human ovarian carcinoma A2780 cells. *Proc. Natl. Acad. Sci. USA* **85**:650–653 (1988).
22. Newman, E. M., Y. Lu, M. Kashani-Sabet, V. Kesavan, and K. J. Scanlon. Mechanisms of cross-resistance to methotrexate and 5-fluorouracil in an A2780 human ovarian carcinoma cell subline resistant to cisplatin. *Biochem. Pharmacol.* **37**:443–447 (1988).
23. Soule, H. D., J. Vazquez, A. Long, S. Albert, and M. Brennan. A human cell line from a pleural effusion derived from a breast carcinoma. *J. Natl. Cancer Inst.* **51**:1409–1416 (1973).
24. Bradford, M. 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).
25. Baserga, R., and D. Malamud. *Modern Methods in Experimental Pathology: Autoradiography*. Harper and Row, New York (1969).
26. Whang-Peng, J., S. Perry, and T. Knutsen. Maturation and phagocytosis by chronic myelogenous leukemia cells *in vitro:* a preliminary report. *J. Natl. Cancer Inst.* **38**:969–977 (1967).
27. Swain, S. M., M. E. Lippman, E. F. Egan, J. C. Drake, S. M. Steinberg, and C. J. Allegra. 5-Fluorouracil and high-dose leucovorin in previously treated patients with metastatic breast cancer. *J. Clin. Oncol.* **7**:890–899 (1989).
28. Lockshin, A., and P. V. Danenberg. Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthetase. *Biochem. Pharmacol.* **30**:247–257 (1981).
29. Chu, M. Y., and G. A. Fischer. Comparative studies of leukemic cells sensitive and resistant to cytosine arabinoside. *Biochem. Pharmacol.* **14**:333–341 (1965).
30. Chu, M. Y., and G. A. Fischer. Effects of cytosine arabinoside on the cell viability and uptake of deoxypyrimidine nucleosides in L5178Y cells. *Biochem. Pharmacol.* **17**:741–751 (1968).
31. Harkrader, R. J., T. J. Boritzki, and R. C. Jackson. Potentiation of 1-β-D-arabinofuranosylcytosine in hepatoma cells by 2′-deoxyadenosine or 2-deoxyguanosine. *Biochem. Pharmacol.* **30**:1099–1104 (1981).
32. Chomczynski, P., and N. Sacchi. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162**:156–159 (1987).
33. Ayusawa, D., K. Shimizu, H. Koyama, S. Kaneda, K. Takeishi, and T. Seno. Cell-cycle-directed regulation of thymidylate synthase messenger RNA in human diploid fibroblasts stimulated to proliferate. *J. Mol. Biol.* **190**:559–567 (1986).
34. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
35. Gerlach, J., J. Kartner, D. Bell, and V. Ling. Multidrug resistance. *Cancer Surv.* **5**:25–46 (1986).
36. Pastan, I., and M. Gottesman. Multidrug resistance in human cancer. *N. Engl. J. Med.* **316**:1388–1393 (1987).

37. Arkin, H., T. Ohnuma, B. A. Kamen, J. F. Holland, and S. Vallabhajosula. Multidrug resistance in a human leukemic cell line selected for resistance to trimetrexate. *Cancer Res.* **49**:6556–6561 (1989).
38. Fairchild, C. R., S. P. Ivy, C. S. Kao-Shan, J. Whang-Peng, N. Rosen, M. A. Israel, P. W. Melera, K. H. Cowan, and M. E. Goldsmith. Isolation of amplified and overexpressed DNA sequences from Adriamycin-resistant human breast cancer cells. *Cancer Res.* **47**:5141–5148 (1987).
39. Batist, G., A. Tulpule, B. K. Sinha, A. G. Katki, C. E. Myers, and K. H. Cowan. Overexpression of a novel anionic glutathione transferase in multidrug-resistant human breast cancer cells. *J. Biol. Chem.* **261**:15544–15549 (1986).
40. Rode, W., K. J. Scanlon, B. A. Moroson, and J. R. Bertino. Regulation of thymidylate synthetase in mouse leukemia cells (L1210). *J. Biol. Chem.* **255**:1305–1311 (1980).
41. Cadman, E., and R. Heimer. Levels of thymidylate synthetase during normal culture growth of L1210 cells. *Cancer Res.* **46**:1195–1198 (1986).
42. Jenh, C. H., L. G. Rao, and L. F. Johnson. Regulation of thymidylate synthase enzyme synthesis in 5-fluorodeoxyuridine-resistant mouse fibroblasts during the transition from the resting to growing state. *J. Cell. Physiol.* **122**:149–154 (1985).
43. Jenh, C. H., P. K. Geyer, and L. F. Johnson. Control of thymidylate synthase mRNA content and gene transcription in an overproducing mouse cell line. *Mol. Cell. Biol.* **5**:2527–2532 (1985).
44. Ayusawa, D., K. Takeishi, S. Kaneda, K. Shimizu, H. Koyama, and T. Seno. Isolation of functional cDNA clones for human thymidylate synthase. *J. Biol. Chem.* **259**:14361–14364 (1984).
45. DeWille, J. W., C. H. Jenh, T. Deng, C. J. Harendza, and L. F. Johnson. Construction and expression of mouse thymidylate synthase minigenes. *J. Biol. Chem.* **263**:84–91 (1988).
46. Takeishi, K., S. Kaneda, D. Ayusawa, K. Shimizu, O. Gotoh, and T. Seno. Nucleotide sequence of a functional cDNA for human thymidylate synthase. *Nucleic Acids Res.* **13**:2035–2042 (1985).
47. Imam, A. M. A., P. H. Crossley, A. L. Jackman, and P. F. R. Little. Analysis of thymidylate synthase gene amplification and of mRNA levels in the cell cycle. *J. Biol. Chem.* **262**:7368–7373 (1987).
48. Juliano, R. L., and V. Ling. A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants. *Biochim. Biophys. Acta* **455**:152–162 (1976).
49. Peterson, R. H. F., and J. L. Biedler. Plasma membrane proteins and glycoproteins from Chinese hamster cells sensitive and resistant to Actinomycin D. *J. Supramol. Struct.* **9**:289–298 (1978).
50. Beck, W. T., M. J. Mueller, and L. R. Tanzer. Altered surface membrane glycoproteins in *Vinca* alkaloid-resistant human leukemic lymphoblasts. *Cancer Res.* **39**:2070–2076 (1979).
51. Kartner, N., J. R. Riordan, and V. Ling. Cell surface P-glycoprotein associated with multidrug resistance in mammalian cell lines. *Science (Washington, D. C.)* **221**:1285–1288 (1983).
52. Riordan, J. R., and V. Ling. Genetic and biochemical characterization of multidrug resistance. *Pharmacol. Ther.* **28**:51–75 (1985).
53. Moscow, J. A., and K. H. Cowan. Multidrug resistance. *J. Natl. Cancer Inst.* **80**:14–20 (1988).
54. Gottesman, M. M., and I. Pastan. The multidrug transporter, a double-edged sword. *J. Biol. Chem.* **263**:12163–12166 (1988).
55. Rice, G. C., V. Ling, and R. T. Schimke. Frequencies of independent and simultaneous selection of Chinese hamster cells for methotrexate and doxorubicin (Adriamycin) resistance. *Proc. Natl. Acad. Sci. USA* **84**:9261–9264 (1987).
56. Assaraf, Y. G., A. Molina, and R. T. Schimke. Cross-resistance to the lipid-soluble antifolate trimetrexate in human carcinoma cells with the multidrug-resistant phenotype. *J. Natl. Cancer Inst.* **81**:290–294 (1989).

Send reprint requests to: Edward Chu, Building 10, Room 12N226, National Cancer Institute, Bethesda, MD 20892.