Resistance of Colorectal Cancer Cells to 5-FUdR and 5-FU Caused by Mycoplasma Infection

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Abstract. *Background: 5-Fluorouracil (5-FU) is an antineoplastic drug that targets thymidylate synthase (TS). Tumour cells can develop resistance to anti-TS drugs by a variety of mechanisms including up-regulation of TS protein and alterations in drug uptake and degradation. The possible mechanisms of the observed rapid development of resistance to the pyrimidine analogs 5-FUdR and 5-FU in cultured HCT116 colon cancer cells were investigated. Materials and Methods: Cell survival was determined in resistant and control HCT116 cells treated with 5-FUdR and 5-FU for 7 days. The ability of the cells to take up and metabolize these drugs was determined by Western blotting and [^{3}H]thymidine incorporation. Results and Conclusion: Resistant HCT116 cells were 5- and 100-fold more resistant to killing by 5-FU and 5-FUdR, respectively, than the parental cells and exhibited impaired uptake. Although the HCT116R cells were initially Mycoplasma free, a low level of Mycoplasma contamination was found in these cells after several weeks in culture. Sensitivity to 5-FUdR was restored by treatment with an anti-Mycoplasma antibiotic. Our observations emphasize the need for frequent testing for Mycoplasma contamination in any cell line under investigation for resistance to anti-TS drugs.*

Thymidylate synthase (TS) (EC 2.1.1.45) catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) by 5,10-methylene tetrahydrofolate to produce deoxythymidine monophosphate (dTMP). This constitutes the final step in the sole *de novo* pathway for the synthesis of dTMP (1). Because of its importance in supplying a deoxynucleotide essential for DNA synthesis, TS is the target for chemotherapeutic agents such as the pyrimidine analogs, 5-fluorouracil deoxyribose (5-FUdR) and 5-fluorouracil (5-FU) (2, 3). 5-FUdR and 5-FU are converted to 5-fluoro-deoxyuridine monophosphate (5-FdUMP), the nucleotide analogue of dUMP, which exerts cytotoxicity principally by forming a stable ternary complex with TS and the folate cofactor 5,10-methylenetetrahydrofolate (4, 5). A principal obstacle to the clinical efficacy of fluoropyrimidines is the emergence of drug resistance, which has been explained by a variety of mechanisms. These include increased intracellular levels of TS (6-9), mutated forms of TS with low affinity for 5-FdUMP or the folate cofactor (10-12), increased phosphatase activity resulting in decreased intracellular accumulation of 5-FdUMP (13), decreased folylpolyglutamylation (14, 15), and decreased thymidine kinase activity (16-18). Decreased transport of nucleoside or nucleobase into cells *via* nucleoside/nucleobase transporters is another potential mechanism of drug resistance (19-25).

Some reports have investigated p53 status and DNA repair defects as causes of resistance to fluoropyrimidines. p53-negative cells have been reported to be >10-fold more resistant to 5-FU than p53-positive parental cells. One group of investigators used a colon cancer line, HCT116, in which p53 was knocked out (26) and a second group used a breast cancer line, MCF-7, in which p53 was inactivated following transformation with human papillomavirus E6 protein (27). Both groups concluded that p53 status can alter response to fluoropyrimidines. However, other evidence suggests that resistance to fluoropyrimidines can be independent of p53 status (28, 29). Sensitivity to 5-FU and 5-FUdR can also be strongly influenced by the mismatch repair status of HCT116 cells (30-32).

In the present report, yet another cause of apparent fluoropyrimidine resistance in cultured cells is described.

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Materials and Methods

Cell culture. HCT116, HCT15, HCC2998, HT29, HeLa and SW620 cells were obtained from the American Type Culture Collection (ATCC) (Rockville, MD, USA). HCT116R cells were derived from the HCT116 cells by continuous culture under non-selective conditions. RKO is a colon cancer line originally derived by Brattain *et al*. (33). Normal primary human fibroblasts were obtained from the National Institute of General Medical Science (NIGMS). Cells were cultured as monolayers in standard medium (Dulbecco's modified Eagle's medium; Gibco-Brl, Burlington, Canada) supplemented with 10% fetal calf serum and maintained in a 95% air /5% CO_2 atmosphere at 37˚C. No antibiotic was added to the culture media. Testing for Mycoplasma contamination was performed with a sensitive PCR-based test for the presence of the conserved spacer region between 16S and 23S rRNA genes (ATCC Mycoplasma Detection Kit, V. 2.0). PCR products were resolved on 2% Metaphor agarose gel and viewed after staining with ethidium bromide. Where indicated, the cell lines were treated with the anti-Mycoplasma antibiotic plasmocin (InvivoGen, San Diego, CA, USA), for a period of 3 weeks according to the manufacturer's instructions.

Western blot analysis of TS inhibition. The cells were plated at 5×10^4 cells per well in a 12-well plate in standard medium. The following day, the medium was replaced with medium containing 5-FUdR or 5-FU at the indicated concentrations. After 24 h, the cells were harvested and protein extracts were examined by Western blotting for the presence of the ternary complex. In brief, the cells were lysed in SDS sample buffer (50 mM 3-(*N*-morpholino)propanesulfonic acid, pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol), proteins were resolved on 12% discontinuous SDS-PAGE and electrophoretically transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). Detection of the ternary complex with rabbit anti-human thymidylate synthase (hTS) antiserum (Rockland Immunochemicals, Gilbertsville, PA, USA) was carried out as previously described (34). Horseradish peroxidase (HRP)-conjugated anti-rabbit/anti-mouse secondary antibody ("Polymer") was purchased from Dako (Carpinteria, CA, USA). Rabbit anti-TS antibody was used at a 1:1,000 dilution and incubated with the membrane for 1 h. HRP was detected using the LumiGLO Chemiluminescent Substrate Kit (KPL Incorporation, Gaithersburg, MD, USA).

Incorporation of [^{3}H]thymidine into DNA. Cells were plated at 7.5×10^3 per well in a 96-well plate. After 48 h, 0.5 μCi of [^{3}H]thymidine (25 Ci/mmol) (Amersham Life Sciences, Buckinghamshire, UK) was added to the wells. The cells were then incubated at 37˚C for 18 h, the medium was then removed and the attached cells were washed with phosphate-buffered saline. Cells were lysed with 0.1% SDS and the total cell lysate counted by liquid scintillation using Ecolite (MP Biomedicals, Solon, OH, USA). Under these conditions, >99% of the radioactivity was incorporated into DNA.

Drug sensitivity assays. A growth inhibition assay was used to assess resistance to 5-FUdR. Cells (1×10^3 cells in 1 mL of standard medium) were seeded in a 12-well plate. One day later, the medium was removed and replaced with medium containing the indicated concentrations of 5-FU or 5-FUdR (Sigma-Aldrich, St. Louis, MO, USA). Eight days later, the attached cells were trypsinized and counted using a Coulter counter (Beckman Coulter, Mississauga, ON, Canada). Non-radioactive thymidine uptake was tested indirectly using a clonogenic assay as follows. Three hundred cells were plated in 5 mL of standard medium in a 60-mm dish. The following day, the medium was replaced with medium containing HA (1×10^4 M hypoxanthine, 4×10^{-7} M aminopterin) and thymidine (either 1×10^{-7} or 1×10^{-5} M). After 10 days, the medium was removed and the colonies were fixed and stained with 0.6% (w/v) methylene blue in methanol. Only colonies >1 mm in diameter were counted. The ability of the cells to take up thymidine and resist killing by aminopterin (in the presence of hypoxanthine) was estimated from the number of colonies in the treated plates divided by the number in the control plates.

Results

HCT116R cells are resistant to 5-FUdR and 5-FU cytotoxicity and to ternary complex formation. During the course of experiments examining drug-resistance of HCT116 cells, some cultures unexpectedly became resistant to killing by 5-FUdR and 5-FU (Figure 1). In this example, the HCT116 cells were approximately 100-fold more resistant to 5-FUdR than the normal parental HCT116 (1A) and approximately 5-fold more resistant to 5-FU (1B). To investigate this in greater detail, a single stock of resistant HCT116 cells was established and maintained in culture under non-selective conditions. This culture was frozen in aliquots and designated as HCT116R cells. For each experiment, a freshly thawed aliquot of HCT116R cells was compared to a freshly thawed aliquot of control HCT116 cells.

The ability of the cells to form the ternary complex was examined following 24 h exposure to 1×10^{-7} M and 1×10^{-5} M 5-FUdR (Figure 2A). The majority of TS in the control HCT116 cells exposed to 1×10^{-7} M 5-FUdR was found to be in the ternary complex. In contrast, in the HCT116R cells treated with 1×10^{-7} M 5-FUdR, the formation of the ternary complex was markedly reduced; at this lower drug concentration, less than one-half of TS was present in the form of the ternary complex. However, following treatment with 100-fold higher concentration of 5-FUdR (1×10^{-5} M), nearly all TS was in the ternary complex in both the parental and resistant HCT116 cells. Similar results were seen following exposure to 5-FU; at 10^{-7} M, very little TS was in the ternary complex in the HCT116R cells whereas most of TS was in the ternary complex in the control cells. Treatment of the cells with higher concentrations of 5-FU (1×10^{-6} M or 1×10^{-5} M) led to accumulation of TS in the ternary complex in both cell lines.

Several other human cell lines were tested for their ability to form the ternary complex upon exposure to 10^{-7} M 5-FUdR (Figure 2B). The cell lines tested included normal fibroblasts, HeLa and 6 colorectal cancer cell lines: SW620, HT29, HCT15, RKO, HCC2998 and HCT116. Treatment of

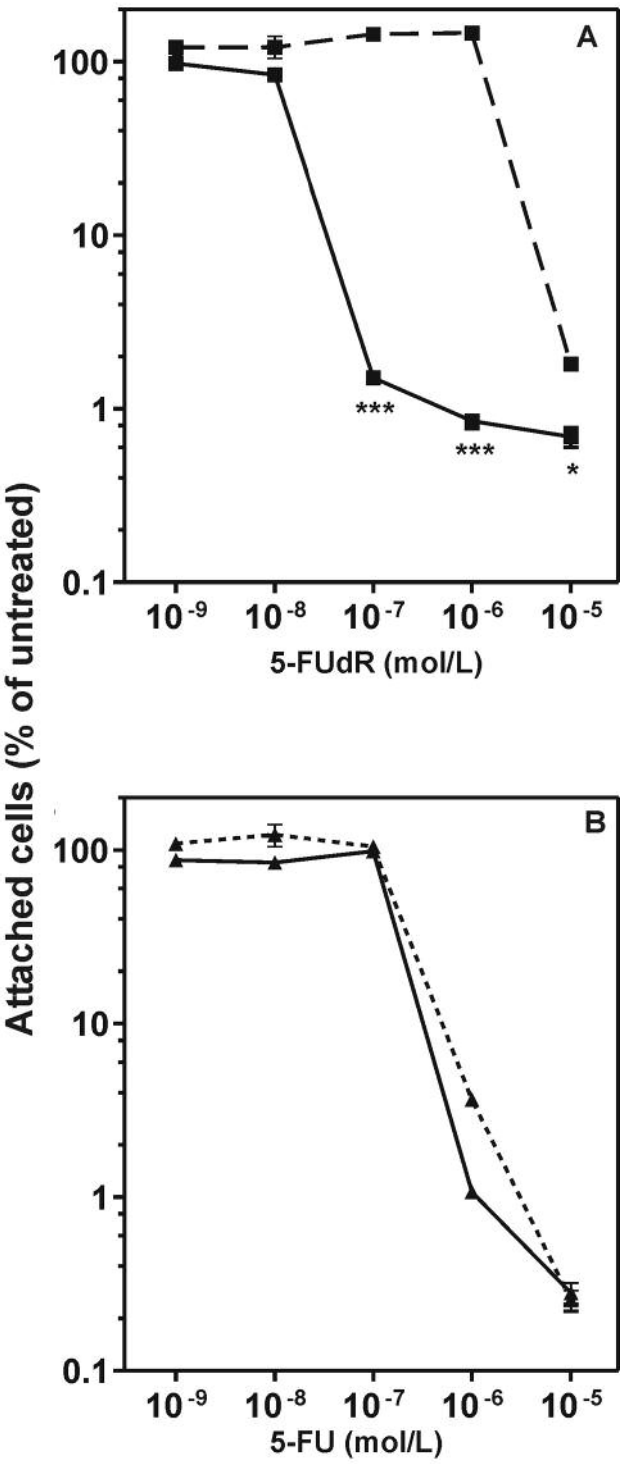

Figure 1. *Dose-dependent effect of 5-FUdR and 5-FU on survival of HCT116 (solid line) and HCT116R cells (dashed line). Cells were treated with various concentrations of 5-FUdR (A) or 5-FU (B) for 8 days. Cells remaining attached to the plate were harvested and counted using a Coulter counter. Cell counts are expressed as percentage of cells in the untreated controls. Error bars represent mean±SD of 3 replicates. Where not shown, error bars are within the symbol. ***p<0.001 and *p<0.05 of one-way ANOVA.*

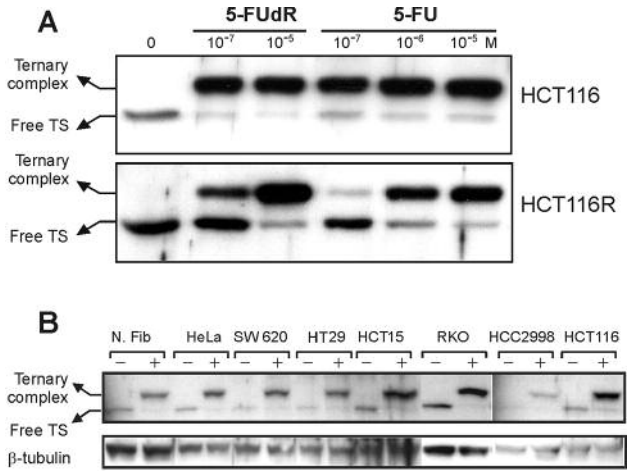

Figure 2. *TS-ternary complex formation following exposure of cells to 5-FUdR or 5-FU. (A) Ternary complex formation following treatment of parental HCT116 and HCT116R cells with increasing concentrations of 5-FUdR and 5-FU for 24 h. Membranes were probed with an anti-TS antibody to detect TS bound to the inhibitor as well as free TS molecules. The position of free TS (~35 kDa) and the ternary complex form of TS (~37 kDa) are indicated. (B) Ternary complex formation following treatment of various cell lines with low concentrations of 5-FUdR. Cells were either not treated or treated with 5-FUdR and analysed by Western blotting.*

all of these cell lines with 1×10^{-7} M 5-FUdR led to the accumulation of TS in the ternary complex, indicating that most cell lines form the ternary complex at relatively low 5-FUdR concentrations.

HCT116R cells and [^{3}H]thymidine incorporation. To determine if the resistance of the HCT116R cells was due to abnormal nucleoside metabolism, the ability of HCT116 and HCT116R cells to incorporate [^{3}H]thymidine into DNA was tested. The HCT116R cells incorporated markedly less [^{3}H]thymidine than did the parental HCT116 cells (Table I).

Ability of thymidine to rescue cells from hypoxanthine and aminopterin toxicity. To confirm that 5-FUdR and 5-FU resistance arose from decreased nucleoside uptake and/or decreased thymidine kinase activity, the parental HCT116 and HCT116R cells were subjected to HAT (hypoxanthine-aminopterin-thymidine) selection. Without added thymidine, very few of either of the cell lines survived the aminopterin treatment (Figure 3). When the culture medium was supplemented with 1×10^{-7} M thymidine, >80% of the parental HCT116 cells were rescued, whereas < 60% of the HCT116R cells were rescued ($p<0.05$). In the presence of higher concentrations of thymidine (1×10^{-5} M), both cultures were completely rescued from aminopterin toxicity.

Table I. *[^{3}H]Thymidine incorporation into HCT116 and HCT116R cells*

	[^{3}H]Thymidine incorporation (counts/min per 10^4 cells)	
	0 h	18 h
No treatment		
HCT116	37±13	26,928±1,091
HCT116R	12±2	300±115**
After plasmocin treatment		
HCT116	N/D	26,738±2,625
HCT116R	N/D	24,894±137

Cells (7.5×10^3) were incubated with 0.5 μCi [^{3}H]thymidine/well. Cell-associated radioactivity was determined by liquid scintillation. Results shown are mean±SEM for 2 to 3 independent experiments. **$p<0.01$, unpaired 2-tailed *t*-test, HCT116 *vs*. HCT116R. N/D, not detected.

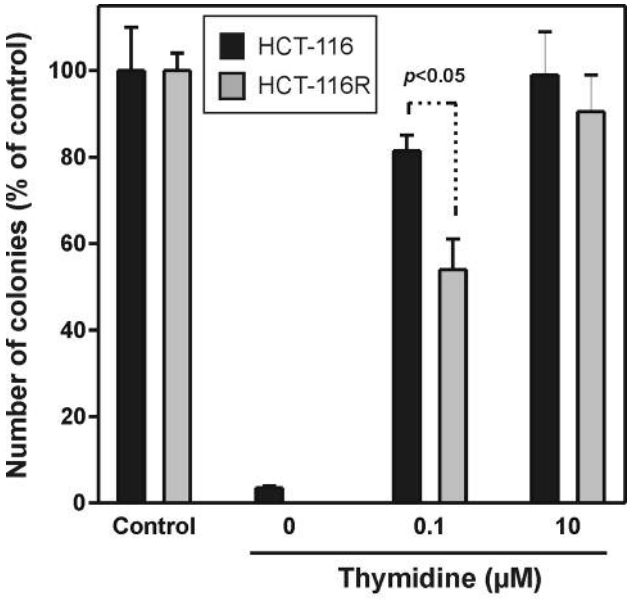

Figure 3. *Effect of hypoxanthine-aminopterin (HA) treatment on colony-formation by HCT116 and HCT116R cells in the presence of increasing concentrations of exogenous thymidine. Cells were either not treated (Control) or treated with HA in the presence of 0, 0.1 or 10 μM thymidine. Colonies were stained with methylene blue and counted after 10 days of treatment. Error bars represent mean±SD of 3 independent experiments.*

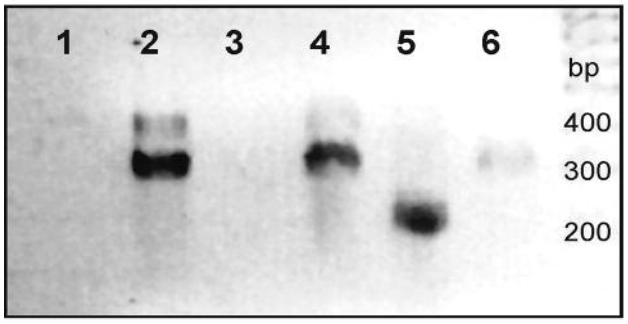

Figure 4. *Detection of Mycoplasma by PCR. Lane 1, negative control (no template); lane 2, HCT116R cells at passage #30; lanes 3 and 4, HCT116 at passage #5 derived from two different frozen aliquots, lane 5, positive control A. Laidlawii; lane 6, positive control M. pirum. PCR products were resolved on 2% Metaphor agarose gel and viewed after staining with ethidium bromide. The assay was conducted according to the kit manufacturer's instructions.*

HCT116R cells and Mycoplasma contamination. Although early passage cell lines used in the present study tested negative for Mycoplasma, this was re-examined because of an earlier report that incorporation of [^{3}H]thymidine is reduced by Mycoplasma contamination (35). Extracts from the cells at different passages (early and late) were prepared and retested for Mycoplasma. Positive amplification was observed for the two controls (*M. pirum* and *A. laidlawii*, supplied with the kit) (Figure 4). Out of two aliquots of HCT116R cells frozen at passage 5, one was Mycoplasma negative and the other positive. Contamination by Mycoplasma was detectable by Hoechst staining for the HCT116R cells at passage 30, but not for the same cells at passage 5 (data not shown).

Treatment with an anti-Mycoplasma antibiotic. To confirm that Mycoplasma contamination was responsible for the observed lower [^{3}H]thymidine incorporation and decreased ternary complex formation, the HCT116R cells were treated for 3 weeks with an antibiotic, plasmocin. The treatment effectively eliminated Mycoplasma in the HCT116R cells as detected by PCR and Hoechst staining (data not shown). Plasmocin treatment also restored the ability of the cells to form the ternary complex following incubation with a low concentration (1×10^{-7} M) of 5-FUdR (Figure 5), as well as the ability of the cells to incorporate [^{3}H]thymidine at a level comparable to the control HCT116 cells (Table I). These results confirmed that impairment of ternary complex formation and [^{3}H]thymidine uptake were phenotypic alterations related to infection of the HCT116R cells with Mycoplasma.

Discussion

The emergence of drug resistance remains the principle obstacle to the successful treatment of malignancies. The original intention of our study was to identify mutations in the TS gene that could confer resistance to the fluoropyrimidines, 5-FU and 5-FUdR, following an earlier study of *in vitro*-

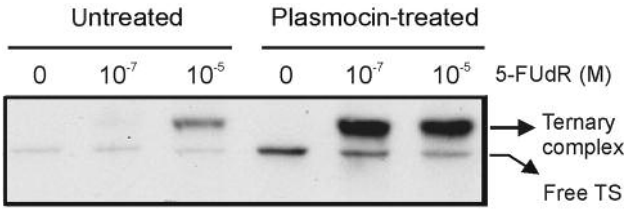

Figure 5. *Effect of plasmocin treatment on ternary complex formation. HCT116R cells were either untreated or treated with plasmocin for 3 weeks according to the manufacturer's instructions. Cells were then incubated with 0, 1×10^{-7} or 1×10^{-5} M 5-FUdR for 24 h and ternary complex formation was analyzed by Western blotting using an anti-TS antibody. The position of free TS (35 kDa) and the ternary complex form of TS (~37 kDa) are indicated.*

induced mutations (36). Unexpectedly, the colorectal cancer line HCT116 used in our study was found to spontaneously acquire resistance to 5-FUdR without exposure to any drug. A routine test for Mycoplasma contamination initially failed to detect its presence and we proceeded to test other possible explanations.

Detection of the ternary complex using the anti-TS antibody is an indication that cells have the ability to take up 5-FU and 5-FUdR, to add deoxyribose to 5-FU and to phosphorylate 5-FUdR to 5-FdUMP. However, the resistant cells failed to form a ternary complex following exposure to low concentrations of 5-FU and 5-FUdR. The search was therefore narrowed to a change in transport, metabolism or binding of 5-FUdR to TS. Uptake and conversion of the nucleosides 5-FUdR and thymidine to their respective nucleotides are mediated by the same cellular proteins. The equilibrative nucleoside transporters (ENT) are responsible for transport of nucleosides into the cell (37), where they are phosphorylated by nucleoside kinase and converted to the corresponding nucleotides (38). No changes in the level of either thymidine kinase or ENT were detected in HCT116R cells (data not shown). Since [^{3}H]thymidine incorporation closely paralleled resistance to the formation of the ternary complex by 5 FUdR, the difference between the HCT116 and HCT116R cells was probably due either to uptake or phosphorylation of the nucleoside. The lower rate of thymidine incorporation in HCT116R was not due to slower growth kinetics since both cultures had similar doubling time in the absence of exogenous thymidine (data not shown). A defect in the metabolism of nucleosides was also indicated by the observation that low concentrations of (unlabelled) thymidine were unable to rescue HCT116R cells treated with hypoxanthine and aminopterin. Aminopterin is a cytotoxic drug that inhibits dihydrofolate reductase (DHFR). Inhibition of DHFR blocks nucleotide synthesis, but cells that possess functional thymidine kinase and phosphoribosyltransferase can be rescued by the addition of exogenous thymidine and hypoxanthine. The HAT assay provided additional evidence that thymidine was either not efficiently taken up or not phosphorylated by thymidine kinase in the HCT116R cells compared to the HCT116 cells.

Mycoplasma contamination can reduce incorporation of [^{3}H]thymidine in cultured cells (39), but Mycoplasma contamination is notoriously difficult to detect (40). Using a PCR-based assay, Mycoplasma was initially undetected in the early passage HCT116 cells, but subsequently it was identified in late passages cell cultures. Treatment of the resistant cell cultures with an appropriate antibiotic eliminated Mycoplasma and reversed the apparent resistance to 5-FUdR, providing strong evidence that the contamination of the culture with Mycoplasms was responsible for the observed resistance.

In summary, Mycoplasma contamination of HCT116 cells can confer resistance to fluoropyrimidines, apparently by preventing uptake of 5-FUdR and 5-FU in an as yet undefined fashion. This resistance closely resembles that described in p53-negative and in mismatch repair-deficient cell lines (26, 30-32). The findings reinforce earlier observations that contamination with Mycoplasma needs to be carefully ruled out, particularly in studies examining cellular resistance to fluoropyrimidines.

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