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 [3H]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 deoxothymidine 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 p55-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.
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 Sciences (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% CO2 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% 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 [3H]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 [3H]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 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 ternary complex in the HCT116R cells whereas most of 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
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 [³H]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 [³H]thymidine into DNA was tested. The HCT116R cells incorporated markedly less [³H]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.
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 ($1 \times 10^{-7}$ 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-
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
uptake or phosphorylation of the nucleoside. The lower rate
of thymidine incorporation in HCT116R was not due 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-FU’dR, to add deoxyribose to 5-FU and to
phosphorylate 5-FU’dR to 5-FdUMP. However, the resistant
cells failed to form a ternary complex following exposure to
low concentrations of 5-FU and 5-FU’dR. The search was
therefore narrowed to a change in transport, metabolism or
binding of 5-FU’dR to TS. Uptake and conversion of the
nucleosides 5-FU’dR 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 [3H]thymidine
incorporation closely paralleled resistance to 5-FU’dR, 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
[3H]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-FU’dR, providing strong evidence that the contamination
of the culture with Mycoplasmas was responsible for the
observed resistance.

In summary, Mycoplasma contamination of HCT116 cells
can confer resistance to fluoropyrimidines, apparently by
preventing uptake of 5-FU’dR 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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