Anti-Cancer Drugs 1998, 9, pp. 625–634

Preclinical study

Comparison between short or long exposure to 5-fluorouracil in human gastric and colon cancer cell lines: biochemical mechanism of resistance

A Harstrick, A Gonzales, N Schleucher, U Vanhoefer, Kun Lu,[1] JL Formento,[2] G Milano,[2] H Wilke, S Seeber and Y Rustum

Department of Internal Medicine, West German Cancer Center, 45122 Essen, Germany. Tel: (+49) 201 723-3100; Fax: (+49) 201 723-5924. [1]Department of Experimental Therapeutics, Roswell Park Cancer Institute, Buffalo, NY 14263, USA. [2]Centre Antoine Lacassagne, 06/89 Nice, France.

Recent preclinal and clinical data indicate that the main mechanisms of 5-fluorouracil (5-FU) cytotoxicity depend on the mode of administration. To gather further insight into the major causes of acquired 5-FU resistance, drug-sensitive human gastric (M2), colon (HT29) and breast (MCF7) cancer cell lines were repeatedly exposed to a fixed concentration of 5-FU given either for 1 or 24 h. Although equieffective doses (IC_{50}) of 5-FU were used, resistance to a 1 h exposure of 5-FU developed faster in all models than to a 24 h exposure. Cell lines with acquired resistance to a 1 h application of 5-FU were only partly cross-resistant to a 24 h exposure, whereas lines with resistance to protracted application of 5-FU displayed significant cross-resistance to the 1 h schedule. Resistance to methotrexate was only seen in cell lines with acquired resistance to 24 h of 5-FU. All 5-FU-resistant cell lines showed reduced incorporation of 5-FU into cellular RNA. Furthermore, elevations of thymidylate synthase were seen in all cell lines with resistance to 24 h of 5-FU but also in one cell line with resistance to a bolus schedule. No alterations in folylpolyglutamate synthase developed in the resistant cell lines. These data support the concept that the main mechanisms of 5-FU cytotoxicity depend on the mode of application. Incorporation of fluorouridine triphosphate into RNA appears to be the most important mechanism of action for 5-FU bolus schedules, whereas inhibition of thymidylate synthase becomes more important as the infusion time is prolonged. These data could have implications on the interaction of 5-FU given at different schedules with various other cytostatic agents. [© 1998 Lippincott Williams & Wilkins.]

Key words: 5-Fluorouracil resistance, colon cancer, gastric cancer, thymidylate synthase inhibition.

Introduction

5-Fluorouracil (5-FU) is one of the most frequently used antineoplastic agents. The drug has documented activity in a variety of human solid tumors, including head and neck cancer, breast cancer, gastric cancer, and colorectal carcinoma.[1-3] Initially, 5-FU was designed to act as a specific inhibitor of thymidylate synthase, the rate-limiting enzyme in pyrimidine *de novo* synthesis.[4] However, subsequent biochemical evaluation demonstrated that 5-FU is extensively metabolized in the cell and can influence a variety of crucial biochemical pathways. After uptake into the cell, 5-FU is converted along various pathways to cytotoxic metabolites and at least three different reactions have been described which can contribute to cytotoxicity: (i) conversion to fluorodesoxyuridine monophosphate (FdUMP), which inhibits thymidylate synthase;[5,6] (ii) metabolization to fluorouracil-triphosphate (FUTP), which is subsequently incorporated into RNA;[7,8] and (iii) incorporation into cellular DNA as fluorodesoxyuridine triphosphate (dFUTP).[9] Currently it is still unclear which of the above-mentioned mechanisms is most important for clinical activity and which biochemical pathways mainly contribute to toxicity.

Clinically, 5-FU is administered in different schedules which differ in their pattern of side effects and might also show incomplete cross-resistance. The classical way of administration has been i.v. push injection, given either weekly or on five consecutive days every 4–5 weeks.[10] With bolus injection, the dose-limiting side effects are myelosuppression and mucositis. If the drug is infused for a prolonged period of time, a significantly higher dose can be administered.[11,12] With the protracted infusion schedule, the

Supported by a grant of the 'Deutsche Forshungsgemeinschaft' (DFG), Bonn, Germany, to AH (HA 1583-2) and a research grant of the DFG to the 'Forschergruppe selektive Tumortherapie TP4' (RA 119–17:1).

Correspondence to A Harstrick

most prominent side effects are diarrhea and hand-foot syndrome. These differences in the pattern of side effects indicate that the metabolism and the mode of action of 5-FU may depend on the administration protocol. Furthermore, it has been demonstrated that clinical resistance to bolus 5-FU can be overcome by protracted infusion schedules.[13,14]

Aschele and co-workers could demonstrate in a human colorectal carcinoma cell line (HCT 8) that the mechanisms of acquired resistance to 5-FU are different when different selection procedures are used. Cells with acquired resistance to a short-term exposure (4 h) displayed resistance by reduced incoporation of FUTP into RNA. However, when cells were made resistant to a continuous exposure of 5-FU (7 days) the main mechanism of resistance appeared to be reduced activity of folylpolyglutamate synthase and consequently insufficient inhibition of thymidylate synthase.[15,16]

Based on the work of Ardalan *et al.*, a 24 h infusion schedule of 5-FU given once a week is frequently used for the treatment of colorectal cancer[11] and is currently compared to bolus 5-FU injection in the ongoing EORTC trial. This 24 h infusion schedule of administration lies somewhat between i.v. bolus injection and protracted infusion over several weeks. In order to gather further insight into mechanisms of acquired resistance to a 24 h exposure in comparison to acquired resistance to a bolus injection, we developed model systems based on 5-FU-sensitive human breast, gastric and colorectal carcinoma cell lines which were evaluated for their mechanisms of resistance. Additionally to biochemical evaluation, the cross-resistance patterns of these 5-FU-resistant to other antimetabilites like methotrexate and tomudex were evaluated.

Materials and methods

Drugs and chemicals

[^{3}H]5-dUMP (sp. act. 18.2 Ci/mmol) and [^{3}H]6-FdUMP (sp. act. 18.0 Ci/mmol) were from Moravek (Brea, CA); [^{3}H]5-FU (sp. act. 25 Ci/mmol) from DuPont (Bad Homberg, Germany). Purified *L caseii* thymidylate synthase was obtained from D Priest (University of South Carolina, Charleston, SC). CH_2PteGlu was a gift from Sapec Fine Chemicals (Lugano, Switzerland), Culture media RPMI 1640 and DMEM were from Seromed (Berlin, Germany), and Leibowitz L-15 from Boehringer (Mannheim, Germany). Tomudex was provided by Zeneca (Macclesfield, UK) and 5-FU by Pharmacia (Erlangen, Germany). All other reagents were from Sigma (Deisenhofen, Germany).

Cell lines

The human colon carcinoma cell line HT29 and the breast carcinoma cell line MCF7 were obtained from the ATCC (Rockville, KD). The human gastric carcinoma cell line M2 was established from ascitic fluid of a patient with advanced gastric adenocarcinoma prior to the initiation of chemotherapy. All cell lines were grown as monolayers in Leibovitz L-15 or RPMI 1640, supplemented with 10% fetal calf serum at 37°C in an atmosphere of 5% CO_2 in air.

Cytotoxicity assay

To determine the activity of the drugs, a modified sulforhodamine B assay as described by Skehan *et al.*[17] was used. In brief, cells were seeded in 96-well microtiter plates and allowed to attach for 24 h. The drugs were added for the given incubation period, the cells were washed and incubated in drug-free medium for an additional 96 h. The medium was removed, the cells were fixed with 10% trichloroucetic acid for at least 1 h, washed five times with tap water, stained with a 0.4% solution of sulforhodamine B in 1% acetic acid, washed five times with 1% acetic acid and the dye was solubilized by the addition of 150 μl Tris buffer (pH 8.5). The absorbance was determined in a Dynatech microplate reader at 570 nm. The concentration of drug to inhibit cell growth by 50% was obtained from semilogarithmic dose-response plots. For all experiments, six wells were used for one drug concentration. All experiments were repeated three times and mean values calculated.

Selection of drug-resistant cell lines

To establish cell lines with acquired resistance to 5-FU, the following selection procedure, which mimics the clinical development of resistance by exposing cells repeatedly to a fixed, not increasing concentration of the drug was used. Wild-type lines MCF7, HT29 or M2 were seeded into 25 cm^2 tissue flasks at a defined density (500 000 cells/flask). The seeding was chosen to allow the cells to reach complete confluence after around 6 days when not treated. To develop resistance the wild-type cells were exposed to 5-FU for either 1 h (R1) or 24 h (R24) starting 24 h after seeding. For each cell line and each given schedule of 5-FU, equieffective concentrations (the individual IC_{50} for the given wild-type cell line) were used (Table 1). No dose escalations were performed during the selection period. After drug exposure, cells were gently washed and incu-

bated with 5 ml of drug-free medium. The cell density was evaluated daily by an inverted phase-contrast microscope and the time to reach confluence was determined for each flask. This time period (passage time) was found to be very constant for all wild-type cell lines (5 days for HT29, 6 days for MCF7 and 6 days for M2). After drug exposure all cell lines showed a significant prolongation of the time needed to reach confluence compared to the non-treated controls. With repeated drug exposure, the passage time of the drug-treated cells declined and finally reached the values of the corresponding wild-type cell lines indicating the development of resistance. Futher drug exposure was performed for an additional two passages after the resistant cell lines had reached the same growth kinetics as the wild-type lines. The resistant lines were then expanded and aliquots were frozen for further analysis. Cell lines made resistant to a 1 h exposure of 5-FU were labled with the suffix 'R1' and cell lines resistant to 24 h of 5-FU as 'R24'.

Enzyme assays

All biochemical assays were performed with cells which had not been exposed to 5-FU for at least 2 weeks. Drug resistance was confirmed at each time using aliquots of the cells which were biochemically evaluated. All assays were repeated four times and mean values were calculated.

Preparation of cell extract. Cells in logarithmic growth were gently trypsinized, washed two times with ice-cold PBS and the dry pellet was resuspended in 100 μl of extraction buffer. The extraction buffer contained 20 mmol/l Tris-HCl, pH 7.5, 250 mmol/l sucrose, 2 mmol/l dithiothreitol, 1.5 mmol/l $MgCl_2$ and 1 mmol/l EDTA. The cells were lysed by alternately freezing in dry ice/ethanol for 5 min and thawing in a 37°C water bath for 5 min. The lysates were centrifuged at 500 *g* for 5 min at 4°C and the supernatant was transferred into 1.5 ml Eppendorf tubes and centrifuged at 8800 *g* for 15 min at 4°C. The second supernatant was transferred into new tubes and kept on ice to be used as cell extract.

Protein concentration. The protein concentration was determined according to the method of Bradford.[18]

Thymidylate synthase catalytic activity. For this assay, the endogenous nucleotides were removed by filtration through centrifugal columns, filled with Sephadex G25, as described by Zhang *et al.*[19] The thymidylate synthase catalytic assay was performed according to Dolnik and Chen.[20] The reaction cocktail contained 220 mmol/l Tris-HCl, 4 mmol/l dithiothreitol, 1.5 mmol/l EDTA, 2.25 mmol/l $MgCl_2$, 150 mmol/l NaF, 4.5 mg/ml BSA, 50 mmol/l CH_2O and 225 μmol/l [^{3}H]5-dUMP (sp. act. 18.2 Ci/mmol).

The reaction cocktail and the cell extract were prewarmed to 37°C and the reaction was started by mixing 10 μl of 4.5 mmol/l $H_4PteGlu_1$ (in 100 mmol/l Tris-HCl, pH 7.5 and 10 mmol/l dithiothreitol), 20 μl ot the reaction cocktail and 30 μl of the filtered cell extract. The reaction tubes were incubated in a 37°C water bath. After 30 min, 200 μl of 10% charcoal (suspended in 2% trichloroacetic acid, ice cold) were added. The mixture was agitated briefly and kept on ice for 15 min, then centrifuged at 8800 *g* for 15 min

Table 1.

	HT29	HT29 R1	HT29 R24	M2	M2 R1	M2 R24
IC_{50} 5-FU 1 h (μM)	456±152	1692±249	4200±855	238±63	848±121	1604±219
RF	–	3.71	9.21	–	3.56	6.74
p value	–	0.0025	0.0021	–	0.028	0.017
IC_{50} 5-FU 24 h (μM)	46±18.6	115±36	212±65	27±11	75.2±29	207±32
RF	–	2.50	4.57	–	2.78	7.67
p value	–	0.032	0.0001	–	0.01	0.004
IC_{50} MTX 24 h (μM)	0.23±0.02	0.25±0.03	0.41±0.02	0.40±0.14	0.26±0.02	0.68±0.08
RF	–	1.09	1.78	–	0.65	1.70
p value	–	0.18	0.04	–	0.12	0.05
IC_{50} tomudex (μM)	6.5±0.5	6.9±0.2	15.2±0.4	1.4±0.5	7.3±1.3	5.0±1.8
RF	–	1.07	2.34	–	5.2	3.6
p value	–	0.12	0.05	–	0.006	0.0017

RF=resistance factor; IC_{50} of resistant cell line divided by IC_{50} of the corresponding wild-type line.
p values were calculated by two-sided t-test comparing IC_{50} of resistant line to IC_{50} of the corresponding wild-type line. IC_{50} values in μM.

at 4°C. Then 100 μl of the supernatant was transferred into 7 ml scintillation vials and counted for radioactivity.

Thymidylate synthase level. A complex binding assay, based on the stoichiometric formation of a covalent ternary complex of thymidylate synthase, 6-[^{3}H]FdUMP and $CH_2H_4PteGlu$ was used. The following components were added to a 1.5 ml tube: 15 μl of 800 nmol/l $CH_2H_4PteGlu_1$ and 15 μl of 830 nmol/l 6-[^{3}H]FdUMP (18 Ci/mmol), 10 μl of 1 mg/ml aprotinin and 60 μl of the filtered cell extract. The mixture was agitated briefly and incubated in a 25°C water bath for 3 h. After 3 h, 50 μl of the reaction solution was transferred into a tube containing 10 μl of 6% SDS. The tubes were agitated, heated at 95°C for 3 min and the ternary complex was separated from unbound 6-[^{3}H]FdUMP by gel centrifugation. The eluents were collected and counted for radioactivity.

Folate levels. The amount of cellular folate was determined by the complex binding assay described by Priest *et al.*[21] The reaction mixture contained *L. casei* thymidylate synthase (65 μU/ml), 6-[^{3}H]FdUMP (125 nmol/l), sodium abscorbate (50 mmol/l), sucrose (213 mmol/l), EDTA (1 mmol/l) and Tris-HCl (50 mmol/l), pH 7.5. The reaction was started by adding 100 μl of cell extract to the reaction mixture in amber colored 1.5 ml tubes. After 60 min in a 25°C water bath, 95 μl of the reaction solution was transferred into tubes containing 5 μl of 20% SDS. The tubes were agitated and heated at 95°C for 3 min. The ternary complexes were separated from unbound FdUMP by passing 25 μl through 400 μl Sephadex G 25 centrifugal columns. The eluents from the columns were collected directly into scintillation vials and counted for radioactivity. The amount of the $CH_2H_4PteGlu$ was calculated based on the specific activity of 6-[^{3}H]FdUMP.

Uptake and retention of methotrexate The influx and efflux kinetics of radiolabeled methotrexate were determined as indirect markers for the uptake of folates and folate polyglutamylation, respectively. For these assays, cells were brought in suspension in growth medium at a concentration of 1×10^6 cells/ml. Cell suspension (1 ml) was transferred into 1.5 ml tubes and incubated at 37°C in a shaking water bath. Uptake was started by addition of radiolabeled methotrexate (final concentration 10 μM). Samples were taken at 1, 5, 10, 20, 30 and 60 min. For determination of drug retention, cells were washed and incubated with drug-free medium for another 2 h with samples taken at regular intervals. Samples were washed two times with ice-cold PBS and the dry pellet was lysed by adding 1 ml of 1 N NaOH. Then 100 μl of the supernatant was removed and transferred into scintillation vials for determination of radioactivity.

Incorporation of FUTP into cellular RNA. Cells in logarithmic phase were seeded at a concentration of 1×10^6 cells/tissue culture dish (Falcon), allowed to grow for 24 h, washed twice with phosphate-buffered saline and then incubated with 4 ml of RPMI-medium containing 5-FU at the individual IC_{50} for the respective wild-type line plus 10 μCi of [^{3}H]5-FU (DuPont; sp. act. 25 Ci/mmol) for 1 and 24 h, respectively. Following drug exposure, cells were washed three times with 5 ml of phosphate-buffered saline and incubated in drug-free RPMI medium for 12 h. Thereafter, cells were harvested with a rubber policeman and washed twice with 5 ml of phosphate-buffered saline. The nucleic acid isolation was performed with Trizol reagent (Gibco/BRL) according to the instruction for total RNA isolation. The mRNA purification was performed with Oligotex mRNA kits (Quiagen). Detection of [^{3}H]5-FU incorporation into RNA was performed by counting the radioactivity of 5 μg of mRNA, in a Beckmann scintillation counter.

Foly-polyglutamate synthase (FPGS) activity assay. Cell line pellets were stored in liquid nitrogen until analysis. On the day of the assay, the cell pellet was suspended in 1 ml buffer (Tris-HCl 10 mM, EDTA 1.5 mM, dithiothreitol 0.5 mM, Na molybdate 10 mM, trypsin soybean inhibitor 0.2 mg/ml, pH 7.5). The cell suspension was freeze-thawed three times, followed by 3×10 s of sonication in ice, and centrifuged for 30 min at 10 500 *g* (4°C). The supernatant (cytosol) was kept on ice until assayed. Proteins were measured using Coomassie G-250 (Protein Assay Reagent; Biorad, Paris, France) with human serum albumin as standard. FPGS activity was measured according to the method described by Montero and Llorente,[22] and consisted of incorporating an additional [^{14}C]glutamic acid residue into the glutamate chain of aminopterin. Each cytosol was assayed in duplicate. The assay consisted of incubating 100 μl of cytotosl with [^{14}C]-glutamic acid (isotopic dilution, 250 μM final concentration) in a total volume of 250 μl. After 2 h incubation at 37°C, the reaction was stopped by addition of 50 μl of 40% trichloracetic acid. Tubes were then centrifuged for 10 min at 3000 *g*. The supernatant was analyzed for the presence of aminopterin polyglutamates by HPLC using an

RP18 5 μm Lichrospher column and a radioactive flow monitor (LD 506; Berthold, Wildbad, Germany). Results are expressed as pmol/h/mg protein. The limit of sensitivity was 25 pmol/h/mg protein. Interassay reproducibility was evaluated through repeated analysis of single-use aliquots of a cell line cytosol (n=5; CV=9.45%).

Results

Development of resistance

To develop cell lines with acquired resistance, cells were repeatedly exposed to the concentration of 5-FU (either for 1 or 24 h) which reduced cell growth to

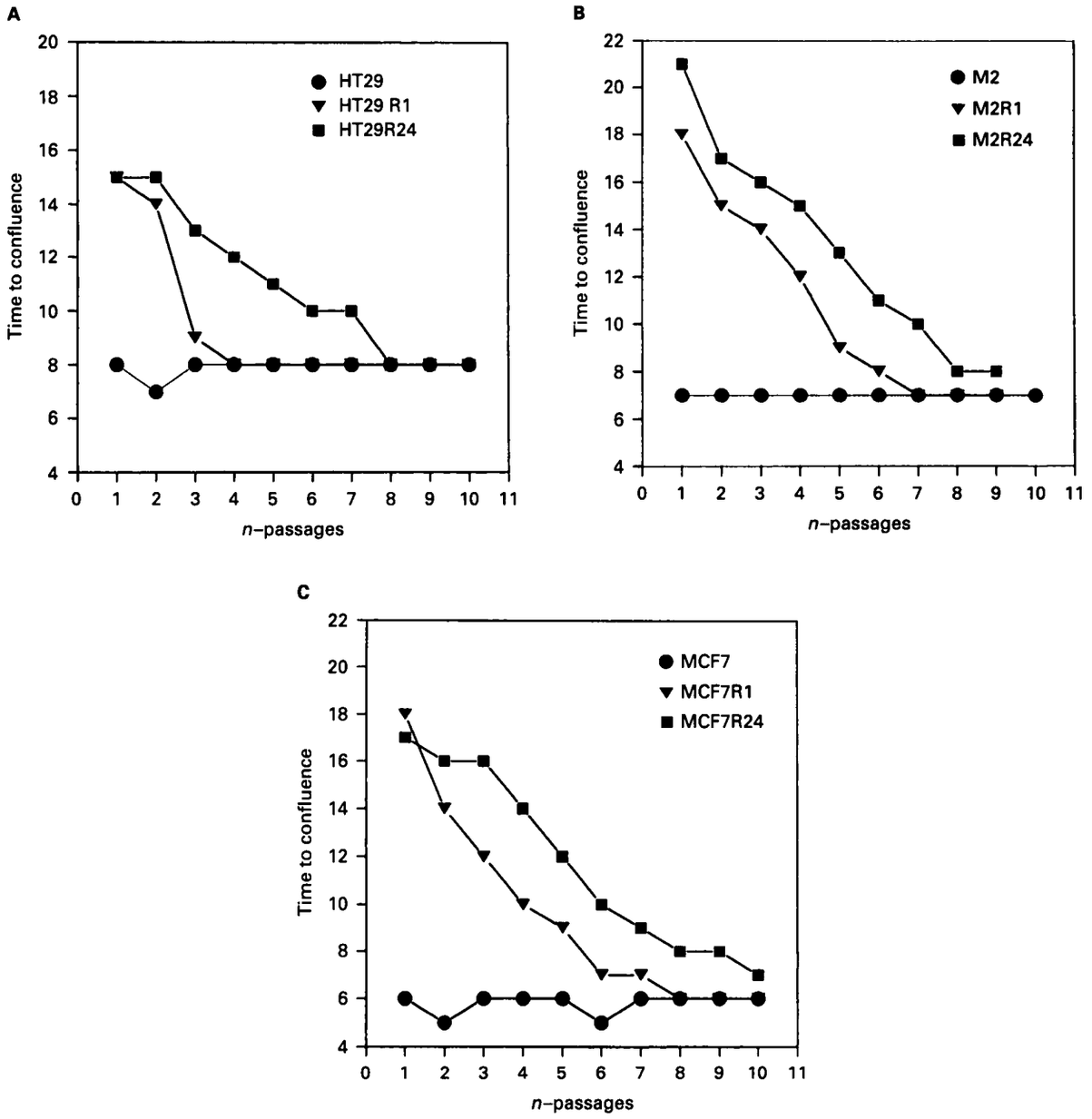

Figure 1. Kinetics of the development of acquired resistance to either a 1 or 24 h incubation of 5-FU. Cells were seeded and treated 24 h later with the IC_{50} of 5-FU. Flasks were then evaluated daily and the time recorded which was needed by the treated cells to grow to 100% confluence (passage time). Cells were then trypsinized, transfered into new flasks and the treatment was repeated until the passage time of drug-treated cells equaled that of untreated controls. As can be seen, resistance to 1 h exposure developed faster in cell lines compared to 24 h exposure. (A) Line HT29. (B) Line M2. (C) Line MCF7.

50% in wild-type cells compared to untreated controls (IC_{50}). The development of resistance was monitored by recording the length of passage time (number of days until cells become confluent). We assumed that cells had became resistant when their passage time after drug exposure equaled that of the corresponding, untreated wild-type cells. Although equieffective concentrations of 5-FU were used (IC_{50} for each line), resistance to a 1 h application developed faster in all cell lines than resistance to a 24 h incubation. The number of passages until resistance had developed were 4, 7 and 5 for lines HT29, M2 and MCF7 after 1 h exposure. The corresponding numbers of passages to develop resistance to 24 h of 5-FU were 8, 9 and 9, respectively. The development of resistance is graphically shown in Figure 1(A-C).

Stability and degree of resistance

To assess the stability of the acquired resistance, cell lines were further grown without drug challenge. The acquired resistance to 5-FU in cell line HT29 R1 and HT29 R24 and in cell line M2 R1 and M2 R24 was stable without further drug exposure for at least 30 passages (representing approximately 3 months). In contrast the resistance in breast carcinoma cell line MCF7 R24 was rapidly lost and the cells regained sensitivity to 5-FU after only 10 passages. Because of this instability of acquired resistance, MCF7 R1 and MCF7 R24 cell lines were not further evaluated.

Cross-resistance

The four resistant cell lines HT29 R1, HT29 R24, M2 R1 and M2 R24 were evaluated for cross-resistance to various schedules of 5-FU and other antimetabolites. The results are summarized in Table 1. Both cell lines selected for resistance by 24 h incubation (HT29 R24 and M2 R24) showed significant resistance to the selection schedule of 5-FU (24 h) as well as to the 1 h application (resistance factors 9.21 and 6.74, respectively). However, cell lines with acquired resistance to 1 h incubation appeared to be not completely cross-resistant to 24 h of 5-FU (resistance factors for 24 h of 5-FU were only 2.50 and 2.78 for lines HT29 R1 and M2 R1, respectively) (Table 1).

Cell lines HT29 R24 and M2 R24 displayed low levels of cross-resistance to methotrexate [resistance factors 1.78 (p=0.04) and 1.70 (p=0.05), respectively], whereas no resistance to methotrexate was seen in both R1 lines. A different pattern of cross-resistance was observed for tomudex, a specific, folate-based inhibitor of thymidylate synthase. Both M2 lines were highly cross-resistant to this drug, whereas no resistance was seen in HT29 R1 and only borderline resistance in HT29 R24 cells.

Biochemical characterization of 5-FU resistance

The biochemical changes in the drug-resistant cell lines are summarized in Tables 2 and 3. The colon cancer cell line HT29 had higher baseline levels of thymidylate synthase than the gastric carcinoma line M2. Both lines with acquired resistance to a 24 h incubation of 5-FU showed significantly elevated levels of thymidylate synthase (complex binding assay and catalytic activity) compared to their corresponding wild-type lines. Also line HT29 R1 displayed a significant elevation of thymidylate synthase, whereas no changes were seen in M2 R1 (Table 2).

No changes in the cellular folate pools were detectable in both 5-FU-resistant colorectal cancer cell lines. In contrast, lines M2 R1 and M2 R24 displayed

Table 2. Thymidylate synthase level, thymidylate synthase catalytic activity, FPGS activity and cellular folate pools in 5-FU-sensitive and 5-FU-resistant cell lines

Line	Thymidylate synthase content (FdUMP-binding) (pmol/mg protein)	p	Thymidylate synthase catalytic activity (pmol/min/mg protein)	p	Folate pools (pmol/mg protein)	p	FPGS activity[a] (pmol/h/mg protein)
HT29	4.72±2.4	–	42.7±14.4	–	28.34±10.9	–	205.5
HT29 R1	16.3±9.9	0.004	177.5±91.4	0.0047	30.8±9.0	0.36	192.3
HT29 R24	12.5±5.1	0.009	136.2±80.7	0.007	31.7±15.4	0.15	175.5
M2	0.81±0.56	–	14.8±10.7	–	26.9±9.6	–	126.8
M2 R1	0.71±0.31	0.37	14.2±8.4	0.40	16.8±2.8	0.053	197.2
M2 R24	1.83±1.06	0.02	39.9±18.2	0.008	17.9±3.7	0.064	118.8

[a]Experiments were performed in duplicate only.

reductions of their cellular folate pools compared to the M2 wild-type line which reached borderline significance in both cases.

The amount of incorporation of FUTP into cellular mRNA is summarized in Table 3. All four 5-FU-resistant cell lines incorporated less FUTP into their mRNA than the drug-sensitive wild-type lines. These differences were statistically significant for three lines (HT29 R24, M2 R1 and M2 R24). In separate eperiments, the uptake of 5-FU into the cells was evaluated. No differences were found between drug-sensitive and drug-resistant lines, which rules out differences in membrane transport as a cause for reduced incorporation of FUTP into RNA.

Table 3. Incorporation of [^{3}H]5-FU into cellular mRNA in 5-FU-sensitive and 5-FU-resistant cell lines (1 h 5-FU) (mean value of four experiments ± SD)

Line	c.p.m./5 μg mRNA	Percent (compared to wild line)	p
HT29	135±23	–	–
HT29 R1	84±20	62.2	0.05
HT29 R24	73±17	58.4	0.040
M2	243±34	–	–
M2 R1	152±29	62.5	0.036
M2 R24	144±37	59.2	0.044

The influx and efflux kinetics of radiolabeled methotrexate are shown in Figure 2(A and B). There were no differences in drug uptake between drug-sensitive and drug-resistant cells. The retention of radiolabeled methotrexate was measured over a 2 h period after incubation in drug-free medium. Again, no differences in the amount of radioactivity retained in the cells were observed between drug-resistant and wild-type cell lines. The results make it unlikely that significant changes in FPGS activity have developed in the drug-resistant cell lines.

FPGS activity was measured in cytosol using the polyglutamylation of aminopterin. As indicated in Table 2, no significant changes in enzyme activity could be detected between drug-sensitive and drug-resistant cell lines, which is in concordance with the data obtained from the methotrexate retention studies.

Discussion

5-FU is one of the most frequently used cytotoxic drugs with documented activity in a variety of malignancies, most notably in breast carcinoma and gastrointestinal tract cancers. 5-FU is cytotoxic to the

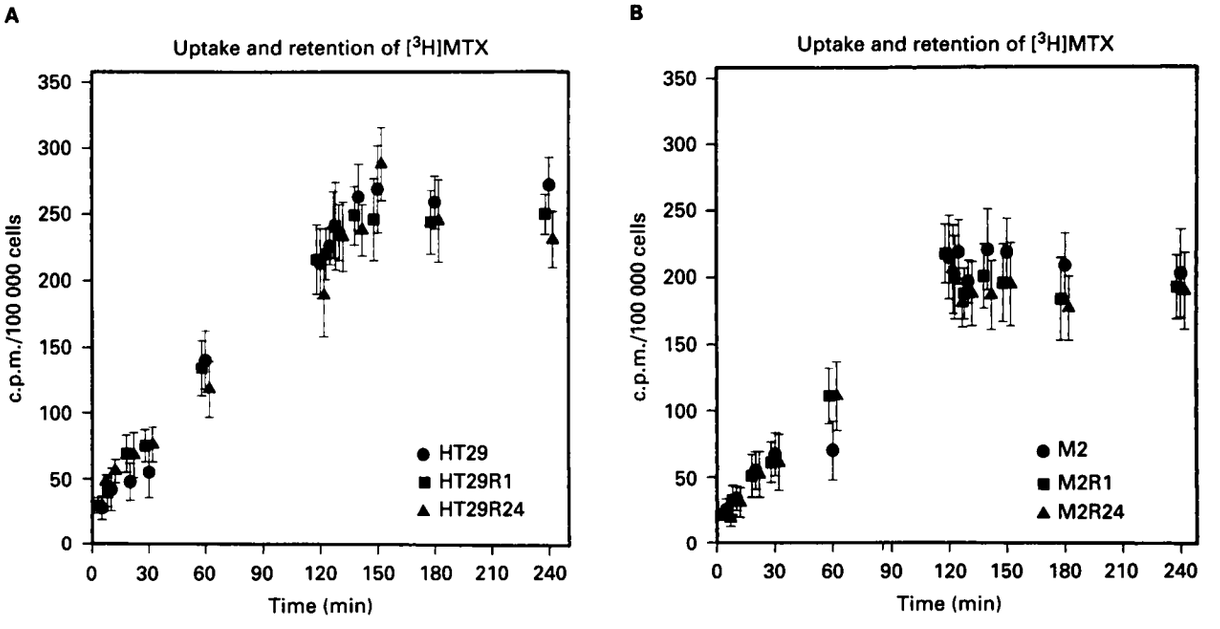

Figure 2. Uptake and retention of [^{3}H]methotrexate in 5-FU sensitive and 5-FU-resistant cell lines derived from human colon (HT29) (A) and gastric (M2) (B) cell lines. Cells were incubated in medium containing 10 μM [^{3}H]methotrexate for 2 h, washed and reincubated in drug-free medium for an additional 2 h. Radioactivity was measured after cell lysis by 1 N NaOH.

cell by at least two different mechanisms: incorporation of FUTP into cellular RNA and inhibition of thymidylate synthase.[4,7,23] Direct incorporation of dFUTP into DNA is a further mechanism which might contribute to cytotoxicity; however, the clinical importance of this pathway has not been elucidated so far.

The mechanisms of 5-FU resistance which were identified in model systems and clinical material are multiple and encompass changes in the anabolizing or catabolizing enzymes, increased levels of dUMP which will compete with FdUMP for the binding to thymidylate synthase, reduced activity of FPGS, altered binding affinity of thymidylate synthase, and reduced incorporation into RNA.[24-29]

However, knowledge about the mechanisms of resistance in relation to various administration schedules of 5-FU is limited. Recently, Aschele *et al.* developed two colorectal carcinoma cell lines resistant to different schedules of 5-FU. They could demonstrate that the mechanisms of 5-FU resistance differed, depending on the selection procedure. In cells made resistant to short-term exposure of 5-FU (4 h), reduced incorporation of FUTP into RNA appeared to be the major mechanism of resistance. Furthermore, these cells were still sensitive to a 7 day exposure of 5-FU. In contrast, cells with acquired resistance to a prolonged exposure of 5-FU over 7 days were not only resistant to the selection schedule but also to short-term exposure (4 h). The major mechanism of resistance in these cells appeared to be a reduction in FPGS activity and the rapid recovery of thymidylate synthase activity, whereas 5-FU uptake and FUTP incorporation into cellular RNA were not affected.[15,16]

Since a 24 h infusion of 5-FU has become a frequently used schedule of administration in Germany and several other European countries, demonstrating clinical activity even in pretreated colorectal cancer patients,[14,20] we sought to establish a model system for acquired resistance to this schedule and to elucidate the mechanisms of resistance. To establish resistance *in vitro*, drug-sensitive cells were repeatedly exposed to a fixed dose of 5-FU which resulted in the development of stable resistance in four cell lines originating from colorectal and gastric cancer. In concordance with the finding of Aschele *et al.*, there was no complete cross-resistance between the two schedules of 5-FU. Although cell lines with acquired resistance to the 1 h application displayed some degree of cross-resistance to the 24 h exposure of 5-FU, the degree of resistance was low (only 2.5- and 2.7-fold, respectively). This is reflected in the clinical experience that patients who have developed resistance to a bolus application of 5-FU might still respond to a 24 h infusion.[13,14] On the other hand, both cell lines with resistance to a 24 h administration were highly cross-resistant (9.2- and 6.7-fold, respectively) to a 1 h exposure. A recent German cooperative study has demonstrated a high response rate (39%) and an encouraging long survival time for chemotherapy naive patients treated with a 24 h infusion of 5-FU in combination with high-dose leucovorin.[31] However, patients progressing on this treatment have not routinely been treated with a bolus schedule and thus clinical experience concerning the activity of a bolus schedule after failure to a 24 h infusion is not available at present.

Both R24 lines, but not the R1 lines, showed low levels of cross-resistance to methotrexate. Furthermore, both R24 cell lines and also M2 R1 cells displayed significant cross-resistance to tomudex, a new folate-based thymidylate synthase inhibitor. Preliminary results of tomudex given to colon cancer patients after failing 5-FU-based therapy also indicate cross-resistance.[31]

The mechanisms of resistance were multifactorial in all cell lines. Most notably, all four 5-FU-resistant cell lines showed statistically significant reductions of FUTP incorporation into mRNA. This is different from the observation made by Aschele *et al.*[15] In their cell lines, FUTP incorporation into RNA was only decreased in the cells resistant to 4 h of 5-FU but remained unchanged in cells with acquired resistance to a 7 day exposure. Cellular thymidylate synthase levels were significantly higher in both R24 cell lines and in HT29 R1 cells compared to the corresponding wild-type cell lines. An interesting, up to now unexplained finding is the fact that this elevation of thymidylate synthase in line HT29 R1 did not result in cross-resistance to tomudex. A possible explanation could either be reduced binding affinity of thymidylate synthase or formation of tomudex polyglutamates in the resistant cell line. Further studies assessing the affinity of isolated enzymes from the resistant cells are in progress.

Significant alterations in the cellular folate levels were seen for both 5-FU-resistant M2 cell lines. Folate pools were reduced to 62.4 and 66.5% in lines M2 R1 and M2 R24, respectively.

If retention of radiolabeled methotrexate after incubation of cells in drug-free medium is taken as an indirect marker for activity of FPGS, there appeared to be no differences between drug-sensitive and drug-resistant cells; findings which were supported by the direct determination of FPGS activity. Thus, unlike in the cell line with acquired resistance to a 7 day exposure which has been described by Aschele *et al.*, alterations in FPGS did not contribute to resistance to a 24 h application of FU in this model.

These data support the concept that the major mechanism of 5-FU cytotoxicity varies with different schedules of application.[32] If 5-FU is given as bolus injection, incorporation into RNA appears to be the major mechnism of cytotoxicity. Consequently, mechanisms of resistance consist of reduced incorporation of FUTP into RNA as demonstrated by Aschele and our current work. If the exposure time of 5-FU is prolonged, there appears to be a gradual shift from RNA-mediated cytotoxicity to inhibition of thymidylate synthase. While mechanisms affecting the inhibition of thymidylate synthase were the only biochemical alterations detectable in cells with resistance to 5-FU given over several weeks, indicating that thymidylate synthase-mediated cytotoxicity might be the only mechanism of action for these prolonged exposure times, our data indicate that the 24 h exposure appears to kill cells by RNA and thymidylate synthase-mediated mechanisms. Consequently, both R24 cell lines displayed mechanisms of resistance that affected RNA incorporation and thymidylate synthase inhibition clinically. This shift in mechanism of action results in a completely different pattern of side effects. Myelosuppression and oral mucositis are the most prominent side effects of bolus injection, whereas mucositis and diarrhea are dose limiting for the 24 h infusion, and diarrhea and hand-foot syndrome become the major side effects for a protracted infusion schedule over several weeks.

Therefore 5-FU probably has to be regarded as being two different drugs depending on the administration schedule. If so it may be rational to combine 5-FU given as bolus and protracted infusion in order to take advantage of these two different modes of action. Laboratory and clinical evidence have been reported to support this concept. Sobrero *et al.* could show that combining 5-FU as short-term exposure (4 h) and as protracted exposure (7 days) results in synergistic antitumor activity.[32,33] Furthermore, the treatment schedule developed by DeGramont *et al.* combining bolus injection and protracted infusion has demonstrated significant antitumor activity, and has been proven to be superior compared to a standard 5-FU bolus regimen in advanced colorectal cancer.[3,4] Additional studies will have to determine whether these changes in the mode of action with different infusion schedule will also have impact on the activity and interactions of 5-FU when given in combination protocols with other cytostatic agents.

Acknowledgments

The authors thank Dr GF Peters, Free University of Amsterdam, for his very helpful discussions; Mrs Heike Druyen and Mrs Beate Lindtner for excellent technical assistance, and Mrs Heike Lunczyn for the skillful preparation of the manuscript.

References

1. Arbuck SG. Overview of clinical trials using 5-fluorouracil and leucovorin for the treatment of colorectal cancer. *Cancer* 1989; **63**: 1036–44.
2. Grem JL. Fluorinated pyrimidines. In: Chabner BA, Collins JM, eds. *Cancer chemotherapy: principles and practice*. Philadelphia: Lippincott 1990: 180–224.
3. Pinedo HM, Peters GFJ. Fluorouracil: biochemistry and pharmacology. *J Clin Oncol* 1988; **6**: 1653–64.
4. Danenberg PV, Lockshin A. Fluorinated pyrimidines as tight-binding inhibitors of thymidylate synthetase. *Pharmacol Ther* 1981; **13**: 69–90.
5. Danenberg PV, Langenbach RJ, Heidelberger C. Structures of reversible and irreversible complexes of thymidylate synthetase and fluorinated pyrimidine nucleotides. *Biochemistry* 1974; **13**: 926–33.
6. Santi DV, McHenry CS, Sommer H. Mechanism of interaction of thymidylate synthetase with 5-fluorodeoxyuridylate. *Biochemistry* 1974; **13**: 471–81.
7. Kufe DW, Major PP. 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. *J Biol Chem* 1981; **256**: 9802–5.
8. Spears CP, Shani J, Shahinian AH, Wolf W, Heidelberger C, Danenberg PV. Assay and time course of 5-fluorouracil incorporation into RNA of 'L1210/0' ascites cells *in vivo*. *Mol Pharmacol* 1985; **27**: 302–7.
9. Lönn U, Lönn S. DNA lesions in human neoplastic cells and cytotoxicity of 5-fluoropyrimidines. *Cancer Res* 1986; **46**: 3866–70.
10. Poon MA, O'Connell MJ, Moertel CG, *et al.* Biochemical modulation of fluorouracil: evidence of significant improvement of survival and quality of life in patients with advanced colorectal carcinoma. *J Clin Oncol* 1989; **7**: 1407–17.
11. Ardalan BA, Chua L, Tian EM, *et al.* A phase II study of weekly 24-hour infusion with high dose fluorouracil with leucovorin in colorectal carcinoma. *J Clin Oncol* 1991; **9**: 625–30.
12. Lokich JJ, Ahlgren JD, Gullo JJ, Philips JA, Fryer JG. A prospective randomized comparison of continuous infusion fluorouracil with a conventional bolus schedule in metastatic colorectal carcinoma: a Midatlantic Oncology Program study. *J Clin Oncol* 1989; **7**: 425–32.
13. Jäger E, Klein O, Wächter B. Second line treatment with high dose 5-fluorouracil and folinic acid in advanced colorectal cancer refractory to standard dose 5-fluorouracil therapy. *Oncology* 1995; **52**: 470–3.
14. Weh HJ, Wilke HJ, Dierlamm J, *et al.* Weekly therapy with folinic acid (FA) and high-dose fluorouracil (5-FU) 24-hour infusion in pretreated patients with metastatic colorectal carcinoma. A multicenter study by the Association of Medical Oncology of the German Cancer Society (AIO). *Ann Oncol* 1994; **5**: 233–7.
15. Aschele C, Sobrero A, Faderan MA, Bertino JR. Novel mechanism(s) of resistance to 5-fluorouracil in human colon cancer (HCT-8) sublines following exposure to two different clinically relevant dose schedules. *Cancer Res* 1992; **52**: 1855–64.

16. Wang FS, Aschele C, Sobrero A, Chang YM, Bertino JR: Decreased folylpolyglutamate synthetase expression: a novel mechanism of fluorouracil resistance. *Cancer Res* 1993; **53**: 3677-80.
17. Skehan P, Storeng R, Scudiero D, *et al.* New colorimetric cytotoxicity assay for anticancer drug screening. *J Natl Cancer Inst* 1990; **82**: 1107-12.
18. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal Biochem* 1976; **72**: 248-54.
19. Zhang ZG, Harstrick A, Rustum YM. Mechanisms of resistance to fluoropyrimidines. *Semin Oncol* 1992; **19**(suppl 3): 4-9.
20. Dolnick BJ, Cheng YC. Human thymidylate synthetase derived from blast cells of patients with acute myelocytic leukemia. Purification and characterization. *J Biol Chem* 1977; **252**: 7697-703.
21. Priest DG, Doig MT. Tissue folate polyglutamate chain length determination by electrophoresis as thymidylate synthase-fluorodeoxyuridylate ternary complexes. *Methods Enzymol* 1986; **122**: 313-9.
22. Montero C, Llorente P. Single step LC method for determination of folylpolyglutamate synthetase activity and separation of polyglutamates. *Chemotherapy* 1993; **37**: 73-8.
23. Roobol C, De Dobbeleer GBE, Bernheim JL. 5-fluorouracil and 5-fluoro-2'-deoxyuridine follow different metabolic pathways in the induction of cell lethality in L 1210 leukaemia. *Br J Cancer* 1984; **49**: 739-44.
24. Ardalan B, Cooney DA, Jayaram HN, *et al.* Mechanisms of sensitivity and resistance of murine tumors to 5-fluorouracil. *Cancer Res* 1980; **40**: 1431-7.
25. Berger SH, Jenh CH, Johnson LF, Berger FG. Thymidylate synthase overproduction and gene amplification in fluorodeoxyuridine-resistant human cells. *Mol Pharmacol* 1985; **28**: 461-7.
26. Lockshin A, Danenberg PV. Biochemical factors affecting the tightness of 5-fluorodeoxyuridylate binding to human thymidylate synthetase. *Biochem Pharmacol* 1981; **30**: 247-57.
27. Moran RG, Spears CP, Heidelberger C. Biochemical determinants of tumor sensitivity to 5-fluorouracil: ultrasensitive methods for the determination of 5-fluoro-2'-deoxyuridylate, and thymidylate synthetase. *Proc Natl Acad Sci USA* 1979; **76**: 1456-60.
28. Myers CE, Young RC, Chabner BA. Biochemical determinantes of 5-fluorouracil response *in vivo*: the role of deoxyuridylate pool expansion. *J Clin Invest* 1975; **56**: 1231-8.
29. Spears CP, Gustavsson BG, Berne M, Frösing R, Bernstein L, Hayes AA. Mechanisms of innate resistance to thymidylate synthase inhibition after 5-fluorouracil. *Cancer Res* 1988; **48**: 5894-900.
30. Köhne CH, Wike H, Hecker H, *et al.* Interferon alpha does not improve the antineoplastic efficacy of high-dose infusional 5-fluorouracil plus folinic acid in advanced colorectal cancer. *Ann Oncol* 1995; **6**: 461-6.
31. Köhne CH, Schmoll HJ, Wilke H, *et al.* Weekly high dose infusional 5-fluorouracil plus leukovorin vs 5FU plus interferon vs 5FU plus leukovorin plus interferon in advanced colorectal cancer. *Proc Am Soc Clin Oncol* 1995; **14**, abstr 453.
32. Sobrero AF, Aschele C, Bertino JR. Fluorouracil in colorectal cancer—a tale of two drugs: implications for biochemical modulation. *J Clin Oncol* 1997; **15**: 368-81.
33. Sobrero AF, Aschele C, Guglielmi AP, *et al.* Synergism and lack of cross-resistance between short-term and continuous exposure to fluorouracil in human colon adenocarcinoma cells. *J Natl Cancer* 1993; **85**: 1937-44.
34. De Gramont A, Bossel JF, Milan C, *et al.* Randomized trial comparing monthly low-dose leucovorin and fluorouracil with bimonthly high-dose leucovorin and fluorouracil bolus plus continous infusion for advanced colorectal cancer: a French intergroup study. *J Clin Oncol* 1997; **15**: 808-15.

(Received 7 April 1998; revised form accepted 5 May 1998)