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Transcription and Activity of 5-Fluorouracil Converting Enzymes in Fluoropyrimidine Resistance in Colon Cancer *In Vitro*

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ABSTRACT. Cellular resistance to 5-fluorouracil (5-FU) is not completely understood. Since 5-FU shares the pyrimidine pathway with the physiological pyrimidines, we investigated the relationship between fluoropyrimidine metabolism, nucleic acid uptake and cytotoxicity of 5-FU in eight colon tumour cell lines including 5-FU-resistant subclones. The cytotoxicity of 5-FU was increased up to 423-fold when the anabolites 5-fluorouridine (FUrd), 5-fluorodeoxyuridine (FdUrd), and 5-fluorodeoxyuridine monophosphate (FdUMP) were compared with the parent drug *in vitro*. The enzymes uridine phosphorylase and thymidine phosphorylase were predictive for the cytotoxicity of 5-FU in 5/7 cell lines. Inhibition of uridine phosphorylase and thymidine phosphorylase by antisense strategies effectively antagonised 5-FU, abolishing 84% and 79% of its toxicity. The importance of thymidine phosphorylase was supported by a highly restricted enzyme activity in 5-FU-resistant cells. In 5-FU naive cells, a stimulating effect of 5-FU on thymidylate synthase mRNA and ribonucleotide reductase mRNA expression was observed. In these cells, antisense oligonucleotides to ribonucleotide reductase significantly reduced cell growth. Downregulation of ribonucleotide reductase mRNA in 5-FU-resistant subclones suggests different mechanisms in primary and secondary resistance to 5-FU. Most of the intracellular 5-FU was selectively incorporated into RNA (range: 45–91%) and generally spared DNA (range: 0.2–11%). In synthesising our data, we conclude that drug resistance could be overwhelmed through bypassing limiting steps in the activation of 5-FU. In the majority of colonic tumours, the activity of uridine phosphorylase and thymidine phosphorylase may have prognostic relevance for the cytotoxicity of 5-FU *in vitro*. BIOCHEM PHARMACOL **54**;11:1233–1242, 1997. © 1997 Elsevier Science Inc.

KEY WORDS. colon cancer; fluoropyrimidines; resistance; metabolism; enzyme transcription; enzyme activity

A successful therapy of colorectal cancer with the antimetabolite 5-fluorouracil[‖] (5-FU) requires intracellular activation of 5-FU, which shares the pyrimidine pathway with the naturally occurring pyrimidine bases. Activation via the ribonucleotide pathway utilises the enzymes uridine phosphorylase and uridine kinase or, alternatively, orotate phosphoribosyltransferase (OPRT) and RNA-polymerase. The deoxyribonucleotide pathway follows the route thymidine phosphorylase, thymidine kinase, and DNA-polymerase [1]. In combination with folinic acid, a third mechanism of action emerges by inhibition of the enzyme thymidylate synthase, depleting tumour cells of thymidine [2–4].

Several previous studies have focused on enzymes as putative parameters to predict the therapeutic response of colorectal carcinomas to 5-FU [5–8]. Decreased expression of enzymes such as dihydrofolate reductase [9] or thymidine kinase [10] has been shown to confer resistance as well as the degree of inhibition of thymidylate synthase [11]. Considering the initial metabolic activation of 5-FU, the involvement of uridine phosphorylase [12] and thymidine phosphorylase [13] in drug resistance has recently been reported. Susceptibility to 5-FU has been attributed to the formation of 5-fluorodeoxyuridine monophosphate (FdUMP) [14], 5-fluorodeoxyuridine (FdUrd) and fluorinated triphosphates [15, 16]. Finally, incorporation of activated 5-FU into nucleic acids has been the subject of several studies [17–22]. With the exception of thymidylate synthase, none of the mentioned *in vitro* parameters has yet been included in a prospective, randomised clinical trial. Statistically significant differences between responders and nonresponders to 5-FU were observed with respect to free thymidylate synthase [23]. The broad overlapping of both

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‖ *Abbreviations*: dNTPs, deoxyribonucleotide triphosphates; FdUMP, 5-fluorodeoxyuridine monophosphate; FdUrd, 5-fluorodeoxyuridine; 5-FU, 5-fluorouracil; FUrd, 5-fluorouridine; OPRT, orotate phosphoribosyltransferase; RT-PCR, reverse-transcriptase polymerase chain reaction.

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groups, however, did not allow individual prognoses of response or resistance to therapy. Alternatively, the ratio thymidylate synthase/β-actin has been used for quantitation after reverse-transcriptase polymerase chain reaction (RT–PCR) [24]. Considering the variety of parameters influencing 5-FU sensitivity/resistance, it seems very unlikely that a single parameter suffices to reliably predict therapeutic response. Supposing a multifactorial mechanism in cellular 5-FU pharmacology, we addressed individual distinctions among different colon tumours. This hypothesis was tested by systematic analysis of the following parameters in eight colon carcinoma cell lines and in two 5-FU-resistant subclones *in vitro*: i) the impact of the 5-FU anabolism on cytotoxicity; ii) the extent of biochemical modulation by [R,S]-folinic acid, [S]-folinic acid and [R,S]-5-methyltetrahydrofolate; iii) the correlation between cytotoxicity and activity of the 5-FU converting enzymes uridine phosphorylase (EC 2.4.2.3), thymidine phosphorylase (EC 2.4.2.4), and OPRT (EC 2.4.2.10); iv) the induction and suppression of mRNA encoding the enzymes uridine phosphorylase, thymidine phosphorylase, thymidine kinase (EC 2.7.1.21), thymidylate synthase (EC 2.1.1.45), OPRT, and ribonucleotide reductase (ribonucleotide diphosphate reductase, EC 1.17.4.1) after exposure to 5-FU; and v) the incorporation of 5-FU into nucleic acids. Once the dominant role of the 5-FU activating enzymes uridine phosphorylase and thymidine phosphorylase was proven, antisense experiments were used to corroborate their importance in susceptibility to 5-FU.

MATERIALS AND METHODS

Cultivation of Colon Tumour Cells

Tumour cells (obtained from ATCC) were maintained in RPMI 1640 supplemented with 4 mM L-glutamine, 10% heat-inactivated fetal calf serum, and 50 μg gentamicin/mL medium (all reagents from GIBCO BRL) at 37°C in a humidified atmosphere of 5% CO_2:95% air. For experimental purposes, cells from the following lines were harvested in their logarithmic growing phase: CCL 227, CCL 228, $CaCO_2$, HT 29, CCL 220.1, CCL 222, CCL 225 and CCL 247.

Resistant subclones of CCL 227 were generated by continuous exposure of tumour cells to 5-FU. We started with the addition of 1 μM 5-FU under standard cell culture conditions. This concentration was gradually enhanced when the growth rate of the exposed cells was similar to that of 5-FU naive cells. During this period, the cytotoxicity of 5-FU was periodically evaluated until a reproducible IC_{50} was obtained in the MTT-assay.

Cytotoxicity Assay

Five thousand tumour cells were seeded in microtiter plates and grown in the presence of fluoropyrimidines (concentration range: 0.001–1000 μM) under standard culture conditions. After incubation for six days in a CO_2-incubator, the cytotoxicity of fluoropyrimidines was evaluated in triplicate using an MTT-assay (Promega). After the addition of dye, the water insoluble crystals were solubilised overnight and the absorbance was monitored at 570 nm with a reference wavelength of 690 nm (surviving fraction of untreated controls grown to confluence within six days: >90%). The following fluoropyrimidines were assayed: 5-fluorouracil (Serva), 5-fluorouridine (Sigma), 5-fluorodeoxyuridine (Sigma) and 5-fluorodeoxyuridine monophosphate (Sigma).

Enzyme Activity of Uridine Phosphorylase, Thymidine Phosphorylase and Orotate Phosphoribosyltransferase

Cells were harvested and lysed in 50 mM Tris buffer (pH 8.0) containing 5 mM $MgCl_2$ and 80 mM mercaptoethanol (all reagents from Merck) by shock freezing in a dry ice/ethanol mixture (repeated three times). After ultracentrifugation, the supernatant was immediately assayed in 5 mM $MgCl_2$, 10 mM NaF, 80 μM 5-FU (containing [^{3}H]-5-FU with 1 μCi activity; Sigma) in 50 mM Tris buffer (pH 8.0) as previously described [5]. The concentration of the respective cofactors was 4 mM (ribose-1-phosphate for uridine phosphorylase, deoxyribose-1-phosphate for thymidine phosphorylase and phosphoribosyl-1-pyrophosphate for OPRT; all reagents obtained from Sigma).

After incubation for 1 hr at 37°, the reaction mixture was separated by TLC on silicagel (Merck) using chloroform: methanol:glacial acetic acid = 20:4:1. The spots corresponding to fluoropyrimidines were excised, extracted with 1M HCl and counted in a liquid scintillation counter after addition of 10 mL Quicksafe (Zinsser).

Protein Determination

Fifty μL cell suspension were lysed overnight in 950 μL 0.1 M NaOH at 37°. The protein concentration was determined according to the method of Bradford using Coomassie brilliant blue as staining reagent and bovine serum albumin in NaOH as a standard [25].

Incorporation of 5-FU into Nucleic Acids

Two days before the addition of 5-FU, 2.5×10^6 tumour cells were seeded in 150 cm^2 cell culture flasks. Tumour cells were incubated with [^{14}C]-5-FU (Sigma) at concentrations from 0.1–1000 μM for six days under standard cell culture conditions. Cells were washed to remove cellular debris from 5-FU-intoxicated cells (surviving fraction of untreated controls grown to confluence within six days: >90%). Adherent cells were then harvested using 0.25% trypsin, washed twice with PBS and lysed in 0.5 M perchloric acid on ice for 15 min. After two washing steps, RNA was solubilised in 0.3 M KOH, whereas DNA was precipitated with ice-cold perchloric acid at 4°. The RNA concentration in the supernatant was determined photometrically at 258 nm. The DNA pellet was washed twice and dissolved in 0.5 M perchloric acid at 80°. The DNA

TABLE 1. PCR primers used to amplify cDNA encoding key enzymes of the (fluoro)pyrimidine pathway

Enzyme	Oligonucleotide, 5′ to 3′	Position	Accession #	Reference
Uridine phosphorylase	+ strand: TGTAATCCCAGCACTTTGAG	191–210	R22017	27
	− strand: GGGTTTCCATCCATGTTG	273–290		
Thymidine phosphorylase	+ strand: ACAAGGTCAGCCTGGTCCTC	491–510	M63193	28
	− strand: TCCGAACTTAACGTCCACCAC	814–834		
Thymidine kinase	+ strand: CTTACTGCGGGACGCCTTGGAGAG	2–26	K02581	29
	− strand: GGGGCAGCCACACAAAGGAGAGTTC	890–914		
Thymidylate synthase	+ strand: GGGCAGATCCAACACATCCTC	208–228	X02308	30
	− strand: AAGAGCACATACATTTCATTCTCCTCAC	1291–1318		
Orotate phosphoribosyl-transferase	+ strand: ACGCCCGGGGCGCCTGGGAGTTTGAA	59–83	J03626	31
	− strand: TTTCCAGCCAGTGACTTTCAGGAGGACCAC	1596–1625		
Ribonucleotide reductase, small subunit	+ strand: CGCGGGAGATTTAAAGGCTGCTGGAGT	96–125	X59618	32
	− strand: TCAGCCAAGTAAGGGCACATCTTCAGTTCA	1366–1395		
β-Actin	+ strand: CATGTACGTTGCTATCCAGGCTGTG	434–458	X00351	33
	− strand: TTTGTCAAGAAAGGGTGTAACGCAAC	1185–1210		

concentration was evaluated at 266 nm [26]. Aliquots from these extracts were counted in a scintillation counter after addition of 10 mL Quicksafe (incorporation into RNA and DNA). To determine the total cellular uptake of 5-FU, harvested cells were washed twice and lysed directly in 10 mL Quicksafe and counted in a liquid scintillation counter.

Reverse-Transcriptase PCR

Cells from CCL 227 and CCL 228 were harvested, washed with cell culture medium and then exposed to 5-FU under cell culture conditions. mRNA expression of the enzymes uridine phosphorylase, thymidine phosphorylase, thymidine kinase, thymidylate synthase, OPRT, and ribonucleotide reductase was evaluated using RT-PCR in untreated controls and after stimulation with 5-FU. RNA was isolated using Ultraspec RNA (Biotecx). Approximately 10^7 washed cells were lysed in 2 mL Ultraspec and extracted with chloroform. RNA was then precipitated overnight with isopropanol at −20°. After centrifugation (12,000 × g, 10 min at 4°), the pellet was washed twice with 75% ice-cold ethanol. The semi-dry pellet was dissolved in DEPC-treated water, containing 40 U RNasin (Promega, Madison, WI) and 1 mM dithiothreitol. The RNA content was determined photometrically. cDNA was synthesised using murine moloney leukaemia virus reverse transcriptase (MULV-RT, GIBCO BRL) under the following conditions: 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol, 0.5 mM deoxyribonucleotide triphosphates (dNTPs), 0.05 mg bovine serum albumin/mL, 1 μM poly dT-primers (Biomedica), 40 U RNasin, 200 U MULV-RT and 5 μg RNA in a total volume of 20 μL. Samples were incubated 60 min at 40° and then stored at −70°.

PCR Conditions

Amplification of the target sequences was carried out in a total volume of 50 μL containing the following reagents: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM $MgCl_2$, 0.25 μM of the forward and reverse primer (Table 1), respectively, cDNA corresponding to 0.25 μg RNA, 0.2 mM dNTPs, 1 U TAQ-polymerase (all reagents from GIBCO BRL), overlayered with 50 μL mineral oil. A "hot start" protocol was used, adding TAQ-polymerase and deoxyribonucleotide triphosphates (dNTPs) at 64° after denaturation of the target for 4 min at 95°. Thirty-five cycles were accomplished with a denaturation temperature of 93° (1 min), annealing at 64° (1 min) and extension at 72° (1–2 min). After completion of the PCR cycles, the last extension time was 5 min at 72°.

Ten μL of the amplification product were diluted in gel loading solution and subjected to gel electrophoresis (1% agarose, Seakem GTG) in Tris-borate-EDTA buffer, pH 8.0 (89 mM Tris, 89 mM boric acid, 0.2 mM EDTA, containing ethidium bromide). Because no quantitative attempt was undertaken to evaluate the mRNA signals, only the categories "no signal", "minor expression", "expression", and "abundant expression" were estimated. Negative controls were assayed during cDNA synthesis and amplification. A 1.0 kb fragment amplified from λ-DNA served as amplification control, whereas a 777 bp fragment of β-actin confirmed the integrity of RNA in RT-PCR (Table 1).

Incubation of Tumour Cells with Antisense Oligonucleotides

Tumour cells were incubated with antisense oligonucleotides to prevent the translation of the enzymes uridine phosphorylase, thymidine phosphorylase, thymidine kinase, thymidylate synthase, OPRT, and ribonucleotide reductase (Table 2). With regard to a melting temperature of approximately 40°, sequences from the 3′ untranslated region were synthesised as phosphorothioate oligonucleotides (Genset and Vienna Biocenter), strictly avoiding G-quartets. Sequences were chosen based upon their thermodynamic properties and were first tested in RT-PCR experiments. Oligonucleotides were synthesised with deoxyribonucleotides and used to generate cDNA from total RNA. The

TABLE 2. Synthetic phosphorothioate oligonucleotides in antisense orientation to mRNA encoding key enzymes of the (fluoro)pyrimidine pathway

Enzyme	Antisense Oligonucleotide, 5′ to 3′	Position	Accession #	Reference
Uridine phosphorylase	CTTGGTTATTTCTTTAGGATC	147–166	R22017	27
Thymidine phosphorylase	TCCACGAGTTTCTTACTG	780–797	M63193	28
Thymidine kinase	AACAGAAACTCAGCAGTG	1106–1123	K02581	29
Thymidylate synthase	CCTCACTTTGTTCATAACCT	1277–1296	X02308	30
OPRT	TCTAACAAACACCAGTCTCA	1770–1789	J03626	31
Ribonucleotide reductase	GTCACTGCTATGGTAAGTCA	1572–1591	X59618	32

successful cDNA synthesis was confirmed by amplification as described in the PCR conditions. Since all generated oligonucleotides met these requirements, no other oligonucleotides were considered for antisense experiments. In the case of uridine phosphorylase and thymidine phosphorylase, the short 3′ untranslated mRNA sequences were inappropriate for this approach. Thus, oligonucleotides complementary to regions within the coding sequence were chosen. One and 10 μM of the corresponding antisense oligonucleotide were incubated with and without 5-FU at the individual IC_{50} of tumour cell lines. After six days at 37° in a humidified atmosphere of 5% CO_2:95% air, the fraction of living cells was evaluated as described in the MTT-assay.

Mathematical Analysis

The IC_{50} was calculated by fitting the data to a sigmoid curve (nonlinear fitting with the software package Inplot™, GraphPad). The same algorithm was applied to calculate the incorporation of 5-FU into nucleic acids. For the statistical evaluation of cytotoxic effects in antisense experiments, groups were compared using the paired Wilcoxon test (nonparametric analysis).

RESULTS

5-FU Metabolism and Cytotoxicity in Eight Colonic Tumours In Vitro

Highly individual resistance patterns to fluoropyrimidines were observed in eight colon carcinoma cell lines. After exposure for six days, the IC_{50} of 5-FU differed by a factor of 100 (range: 0.3 to 31.6 μM). A similar observation was made when the metabolites of 5-FU were assayed instead of the parent compound. In comparison with 5-FU, the cytotoxic effect of 5-fluorouridine (5-FUrd), 5-FdUrd and 5-FdUMP was up to 423-fold higher (Table 3). Even in HT 29, the most 5-FU sensitive cell line, the ratio IC_{50} 5-FU/IC_{50} FdUrd was approximately 10. In six other colonic tumours *in vitro*, exposure to FUrd instead of 5-FU resulted in more than a 10-fold elevation of the cytocidal effect. Similar ratios were observed comparing 5-FU and FdUrd. Considering absolute values, the lowest IC_{50} was observed after exposure to FdUMP (range: 0.014–0.44 μM) and its precursor FdUrd (range: 0.033–0.95 μM). This complex pattern was then analysed by statistical methods. The activities of 5-FU, FUrd, FdUrd and FdUMP were independent parameters as shown by correlation analysis of the respective IC_{50}. The only exception was a linear relationship between the IC_{50} of FdUrd and FdUMP ($r = 0.82$; $N = 8$), arguing against a decisive role of thymidine kinase as limiting step in the activation of 5-FU.

Biochemical Modulation of Fluoropyrimidines with Tetrahydrofolates

In order to explore therapeutic options with fluoropyrimidines, we evaluated the biochemical modulation of 5-FU and its metabolites. Considering different tetrahydrofolates, the modulation by 10 μM [R,S]-folinic acid, 5 μM [S]-

TABLE 3. Cytotoxicity of 5-FU and its anabolites to colorectal tumor cells *in vitro* after exposure for six days

Cell line	Source*	IC_{50} [μM] 5-FU	5-FUrd	5-FdUrd	5-FdUMP	Ratio†
CCL 227	Met	18.1	0.49	0.21	0.043	423
CCL 228	PT	5.9	0.26	0.086	0.11	69
HT 29	PT	0.31	0.54	0.033	0.12	9.5
CaCo-2	PT	3.6	0.23	0.25	0.026	139
CCL 220.1	PT	31.6	0.57	0.17	0.15	211
CCL 222	PT	—	0.61	0.13	0.014	—
CCL 225	PT	11.2	0.15	0.19	0.22	75
CCL 247	PT	3.1	0.08	0.95	0.44	39

Cells were seeded in triplicate in microtiter plates and incubated with fluoropyrimidines for 6 days under standard cell culture conditions. After 6 days, the fraction of surviving cells was determined by the MTT-assay as described in "Materials and Methods." The IC_{50} was then calculated by nonlinear fitting of the data to a sigmoid curve.

* "PT" refers to a primary tumor; "Met" indicates a cell line established from a lymph node metastasis with CCL 228 as the primary tumor.

† Ratio = IC_{50} of 5-FU divided by the IC_{50} established for the most cytotoxic anabolite.

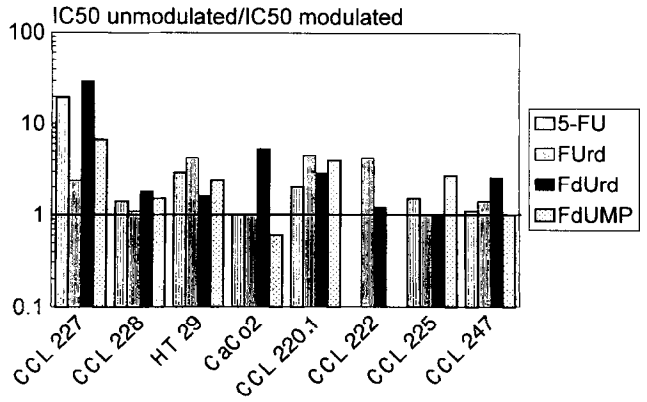

FIG. 1. Modulation of the cytotoxicity of 5-FU and its anabolites by tetrahydrofolates. Bars represent the IC_{50} of tumor cell lines exposed to fluoropyrimidines divided by the IC_{50} after the addition of tetrahydrofolates as evaluated by the MTT-assay (six days of exposure); a ratio = 1 indicates the absence of biochemical modulation. The modulated IC_{50} was calculated as the mean IC_{50} after incubation with fluoropyrimidines and 10 μM [R,S]-folinic acid or 5 μM [S]-folinic acid or 10 μM [R,S]-5-methyltetrahydrofolate, respectively.

folinic acid and 10 μM [R,S]-5-methyltetrahydrofolate, respectively, exhibited very similar activity, although differences up to 3-fold were observed in some cases (data not shown). With the exception of cell lines highly susceptible to modulation (e.g., CCL 227), only moderate responses were achieved by the addition of tetrahydrofolates to fluoropyrimidines after long-term exposure (e.g., CCL 225 and CCL 228). All other cell lines, such as CCL 220.1, showed a fairly constant level of biochemical modulation (Fig. 1).

The extent of the biochemical modulation of 5-FU by tetrahydrofolates differed only sporadically among the cell line investigated (range of the ratio IC_{50} unmodulated/IC_{50} modulated: 1 to 20). The biochemical modulation was active at every step of the fluoropyrimidine metabolism involving the modulation of fluorinated nucleosides as well as fluorinated nucleotides (range of the ratio IC_{50} unmodulated/IC_{50} modulated: 1 to 30).

Activity of 5-FU Converting Enzymes

Given the different sensitivity of tumour cells to fluoropyrimidines, we next evaluated the initial steps of 5-FU anabolism. As a consequence of the different cytotoxic pattern of fluoropyrimidines, a differential activation of 5-FU was expected. In fact, the enzyme activities of uridine phosphorylase, thymidine phosphorylase and OPRT varied by a factor of 10 among the tumours investigated *in vitro* (Table 4). In five out of seven cell lines, there was a statistically significant impact of the initial activation steps of 5-FU on cytotoxicity: the ratio thymidine phosphorylase/uridine phosphorylase was proportional to the ratio IC_{50} of FUrd/IC_{50} of FdUrd ($r = 0.95$; $N = 5$). When considering seven cells lines, the overall correlation dropped to 0.04. All other correlations between the activity of (fluoro)pyrimidine converting enzymes and toxicity were not significant. The direct transfer of ribosylphosphate to 5-FU via OPRT, although an active and important pathway in colorectal cancer cells, had no prognostic importance with regard to cytotoxicity. Due to the fact that no reproducible IC_{50} for 5-FU could be established in CCL 222, statistics were calculated only for seven of the eight cell lines.

End point determinations of the enzymatic activity without addition of the respective phosphorylated sugars cut down the conversion of 5-FU in CCL 227. In comparison with optimal assay conditions, the remaining enzyme activity was in the range of 5% to 22% (Table 5). In contrast to thymidine phosphorylase and OPRT, the addition of ATP severely increased the formation of phosphorylated compounds when assaying uridine phosphorylase (mean factor: 5.3; range: 1.2–13.1).

Suppression of Enzyme Translation by Antisense Oligonucleotides

Phosphorothioate antisense oligonucleotides were used to inhibit the translation of uridine phosphorylase, thymidine phosphorylase, thymidine kinase, thymidylate synthase, and OPRT. Using an MTT-assay, none of the mentioned

TABLE 4. Conversion of 5-FU by the enzymes uridine phosphorylase, thymidine phosphorylase and orotate phosphoribosyltransferase in eight colorectal tumor cell lines

	Enzyme activity (pmol 5-FU conversion/mg protein per min)		
	Uridine phosphorylase	Thymidine phosphorylase	Orotate phosphoribosyl-transferase
CCL 227	14.5 ± 1.3	41.8 ± 3.2	41.2 ± 2.9
CCL 228	21.9 ± 0.9	53.6 ± 2.2	54.3 ± 9.4
HT 29	19.3 ± 0.8	68.6 ± 3.2	68.6 ± 3.1
$CaCO_2$	60.5 ± 2.7	50.3 ± 4.1	39.2 ± 3.1
CCL 220.1	<1.0	21.3 ± 8.1	84.2 ± 1.5
CCL 222	6.2 ± 0.3	45.9 ± 12.8	61.4 ± 1.8
CCL 225	15.0 ± 1.5	13.9 ± 5.8	56.9 ± 2.2
CCL 247	12.1 ± 0.5	100.5 ± 11.0	29.4 ± 1.2

Cells in the logarithmic growing phase were harvested and lysed by shock freezing. The enzyme activities were determined in triplicate from the cell lysate after incubation with radiolabeled 5-FU and separation of the metabolites using thin layer chromatography. The radiolabeled compounds were extracted and counted in a liquid scintillation counter. Enzyme activities are reported as mean ± SD.

TABLE 5. Conversion of 5-FU by the enzymes uridine phosphorylase, thymidine phosphorylase and orotate phosphoribosyltransferase in CCL 227 and two 5-FU resistant subclones

	Enzyme activity [pmol 5-FU conversion/mg protein per min]					
	Uridine phosphorylase		Thymidine phosphorylase		Orotate phosphoribosyl-transferase	
	+ substrate	no substrate	+ substrate	no substrate	+ substrate	no substrate
CCL227	14.5 ± 1.3	2.5 ± 0.1	41.8 ± 3.2	9.2 ± 2.3	41.2 ± 2.9	2.2 ± 0.3
CCL227 + 5 μM 5-FU	14.7 ± 0.3	2.1 ± 0.1	26.3 ± 1.7	6.1 ± 0.5	30.4 ± 1.1	2.3 ± 0.3
CCL227 + 25 μM 5-FU	17.0 ± 0.4	2.4 ± 0.1	12.7 ± 0.5	3.8 ± 0.4	26.9 ± 0.2	1.5 ± 0.4

Cells in the logarithmic growing phase were harvested and lysed by shock freezing. The enzyme activities were determined in triplicate from the cell lysate after incubation with radiolabeled 5-FU with and without substrate (ribose-1-phosphate for uridine phosphorylase, deoxyribose-1-phosphate for thymidine phosphorylase and phosphoribosyl-1-pyrophosphate for OPRT) followed by separation of the metabolites using thin layer chromatography. The radiolabeled compounds were extracted and counted in a liquid scintillation counter. Enzyme activities are reported as mean ± SD.

antisense oligonucleotides alone exhibited a cytotoxic effect (incubation with 1 μM and 10 μM antisense oligonucleotide for 6 days). The corresponding sense controls were also without cytotoxic effect.

A marked inhibition was observed, however, when incubations were done with an antisense oligonucleotide to ribonucleotide reductase mRNA. One μM antisense oligonucleotide did not significantly influence cell growth, whereas the cytotoxic effect of 10 μM antisense oligonucleotide alone (50% growth reduction in comparison with the untreated control) was further enhanced by the addition of 5-FU. As a result, virtually all tumour cells were eradicated by the combination 5-FU and 10 μM antisense oligonucleotide to ribonucleotide reductase mRNA. In both cell lines, the effect of the combination 1 μM antisense oligonucleotide and 5-FU was comparable to that of 5-FU alone (all incubations done for 6 days at the IC_{50} of 5-FU). The inhibition of protein translation by antisense oligonucleotides hybridising uridine phosphorylase mRNA and thymidine phosphorylase mRNA did not change the growth characteristics of the tumour cell lines CCL 227 and CCL 228, nor did the corresponding sense oligonucleotide used as control (concentration: 1 μM and 10 μM). In accordance with the studies underlining the central role of the initial 5-FU metabolism, the cytocidal effect of 5-FU was partly abolished by application of the antisense strategy. In CCL 227, the cytotoxicity of 5-FU at the IC_{50} was reduced in a dose-dependent manner: One micromolar antisense oligonucleotide to uridine phosphorylase reversed the effects of 5-FU by 39% (surviving fraction of cells: 69.5 ± 4.3% vs. 50.0 ± 4.6%; $P < 0.01$) and 10 μM antisense oligonucleotide by 84% (surviving fraction of cells: 92.0 ± 5.1% vs. 50.0 ± 4.6%; $P < 0.01$). Similarly, 1 μM antisense oligonucleotide to thymidine phosphorylase abolished the effect of 5-FU by 23% (surviving fraction of cells: 61.5 ± 0.5% vs. 50.0 ± 1.0%; $P < 0.01$) and 10 μM antisense oligonucleotide by 79% (surviving fraction of cells: 89.5 ± 1.5% vs. 50.0 ± 1.0%; $P < 0.01$). In CCL 228, the antagonising effect was similar, but was less pronounced (reduction of 5-FU cytotoxicity in the range of 20% to 50%; comparison with the IC_{50} of 5-FU: $P < 0.01$). Due to the large intra-assay variation when assaying thymidine phosphorylase, the effect of 1 μM and 10 μM antisense oligonucleotide combined with 5-FU was not significantly different. Considering the complete absence of cytotoxicity of sense and antisense oligonucleotides for both phosphorylases in control experiments, an antagonising effect to 5-FU can be deduced from these experiments. By contrast, a synergistic effect with 5-FU was observed with the ribonucleotide reductase antisense strategy.

Incorporation of 5-FU into Nucleic Acids

As pharmacological endpoint of long-term incubations with 5-FU, we investigated the uptake of radiolabeled 5-FU into nucleic acids. The incorporation of 5-FU into nucleic acids was proportional to the concentration of 5-FU in the medium and could mathematically be described by a sigmoid curve (dose-response relationship). The uptake mainly concerned total RNA after exposure for 6 days with 5-FU. In DNA, incorporated 5-FU was generally detected at low concentrations, indicating that high protective mechanisms were operative even after several days of cytotoxic stress (Fig. 2).

Under *in vitro* culture conditions, the relative cellular content of DNA and RNA (expressed as microgram nucleic

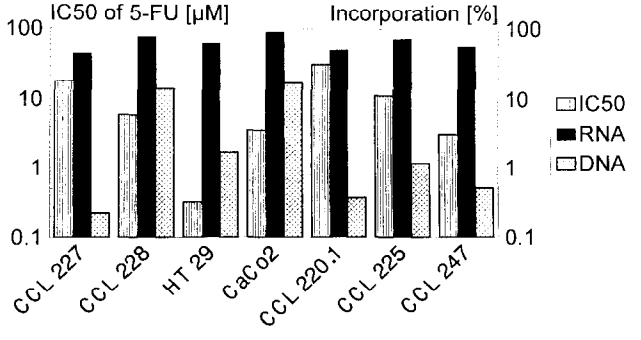

FIG. 2. Comparison of the uptake of 5-FU into nucleic acids at the IC_{50} after exposure for six days. The incorporation of [^{14}C]-5-FU into nucleic acids was determined after extraction of RNA and DNA as described in "Materials and Methods." The incorporation into nucleic acids was calculated as percentage of the cellular uptake of 5-FU.

acid per milligram protein) was not influenced by exposure to 5-FU in concentrations ranging from 0.1–100 μM. No significant correlation was observed between the IC_{50} of 5-FU and cellular uptake or incorporation into nucleic acids. At the respective IC_{50} of 5-FU, the incorporation of radiolabeled 5-FU into nucleic acids varied by a factor of 124 for DNA and a factor of 23 for RNA (expressed as cpm/μg nucleic acid) among the cell lines. The cellular uptake of 5-FU varied by a factor of 20 (expressed as cpm/μg protein). The total balance of radiolabeled parent compound proved RNA to be the major target for 5-FU, whereas DNA incorporation was generally below 2% of the cellular uptake (range: 0.2–11%). The incorporation into total RNA ranged from 45% to 91% of the cellular uptake, leaving only a restricted range for cytotoxic metabolites within cells. In fact, even in CCL 227 (45% of 5-FU incorporated into nucleic acids), neither FUrd nor FdUrd could be detected intracellularly after six days, suggesting a fast catabolic breakdown of unincorporated 5-FU. The presence of 10 μM [R,S]-folinic acid in the medium had no effect on the incorporation of 5-FU at various concentrations (range: 0.1–1000 μM 5-FU) into nucleic acids at the IC_{50}, excluding a synergistic cytotoxic mechanism by enhanced nucleic acid uptake.

Transcription of Fluoropyrimidine Converting Enzymes after Exposure to 5-FU

To understand the regulation of the pyrimidine network, the induction or suppression of enzyme mRNA was investigated after long-term stimulation of CCL 227 and CCL 228 with 5-FU. Cells were exposed to 5-FU at their IC_{50} for seven days. Uridine phosphorylase, thymidine phosphorylase, thymidine kinase, thymidylate synthase, OPRT, and ribonucleotide reductase were then assayed in naive cells on days 1, 2, 4 and 7 after incubation with 5-FU by RT-PCR.

Uridine phosphorylase mRNA and thymidine kinase mRNA were strongly expressed in viable cells and were subject to minor changes during the incubation period. The transcription of thymidine phosphorylase mRNA was unaltered in both cell lines. This was true for the low expression in CCL 227 as well as for the abundant expression in CCL 228. As expected, thymidylate synthase was stimulated in both CCL 227 and CCL 228. OPRT does not seem to play a major role under cell culture conditions: the weak signal showed no significant changes either in the untreated control or in the challenged cells. The low transcription of ribonucleotide reductase mRNA was stimulated by 5-FU, whereas its expression was constant in the controls (Fig. 3). A reduced transcription was observed only for uridine phosphorylase in the untreated controls at the end of the growing phase, whereas all other signals were constantly expressed (Table 6).

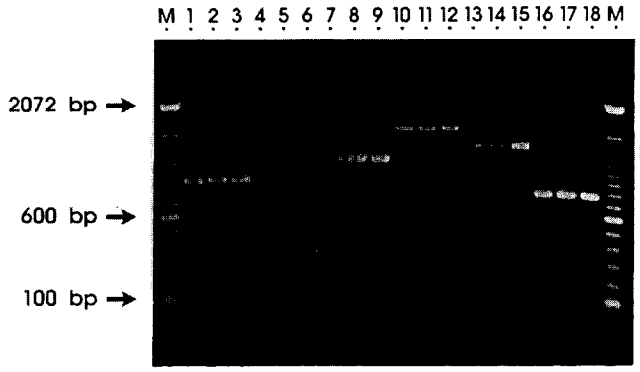

FIG. 3. mRNA kinetics of (fluoro)pyrimidine converting enzymes after exposure to 5-fluorouracil in colorectal tumors *in vitro*. Cells were exposed to 5-fluorouracil at their IC_{50} for seven days. Total RNA was isolated prior to exposure and 1, 2, 4 and 7 days after addition of 5-fluorouracil. Samples were subjected to RT-PCR as described in "Materials and Methods" and were analysed on a 1% agarose gel in TBE-buffer. M = 100 bp-ladder, 1–3 = thymidine kinase (CCL 228 on d0, d2, d7), 4–6 = thymidine phosphorylase (CCL 227 on d0, d2, d7), 7–9 = thymidylate synthase (CCL 228 on d0, d2, d7), 10–12 = orotate phosphoribosyltransferase (CCL 228 on d0, d2, d7), 13–15 = ribonucleotide reductase (CCL 227 on d0, d2, d7), 16–18 = β-actin control (777 bp).

The (Fluoro)Pyrimidine Pathway in Two Resistant Subclones of CCL227

Because resistance to 5-FU is a common feature in the therapy of colorectal tumours, we selected resistant tumour cells by continuous exposure to 5-FU. The transcription and the activity of 5-FU converting enzymes were compared with those of the parent cell line. In two 5-FU-resistant subclones of CCL 227 (cultured at 5 μM and 25 μM 5-FU), the enzyme activity of thymidine phosphorylase was reduced to one third of the activity of the parent cell line (IC_{50}:353 μM 5-FU of the highly resistant cell line vs. IC_{50}:18.1 μM 5-FU for the parent cell line). Irrespective of the suppression of thymidine phosphorylase, the activities of uridine phosphorylase and OPRT showed minor variations. In resistant subclones, the end point determination of enzyme activity without addition of the respective phosphorylated sugars considerably reduced the metabolism of 5-FU. Even with limited substrate, the results were proportional to those obtained under optimal assay conditions (Table 5). Although statistically significant, the suppression of OPRT in the resistant subclones is likely to be of secondary importance, because an alternative pathway to OPRT exists.

As expected after circumvention of the initial 5-FU activation, the cytotoxicity of FUrd was comparable in parent cells and in the resistant subclones. In contrast to the cell line grown under 5 μM 5-FU, the most resistant cell line became fourfold insensitive to FdUrd (IC_{50} of CCL 227: 0.021 μM FdUrd; IC_{50} of CCL 227 + 5 μM 5-FU: 0.026 μM FdUrd; IC_{50} of CCL 227 + 25 μM 5-FU: 0.106 μM FdUrd). In accordance with the induction of thymidy-

TABLE 6. Induction and suppression of (fluoro)pyrimidine converting enzymes under exposure to 5-FU for seven days

	IC_{50} [μM]		Uridine phosphorylase		Thymidine phosphorylase		Thymidine kinase		Thymidylate synthase		OPRT		Ribonucleotide reductase	
	CCL 227	CCL 228	CCL 227	CCL 228	CCL 227	CCL 228	CCL 227	CCL 228	CCL 227	CCL 228	CCL 227	CCL 228	CCL 227	CCL 228
Constitutive expression	—	—	++	++	+	+++	+++	+++	++	++	+	+	+	+
Control	—	—	=	↓	=	=	=	=	=	=	=	=	=	=
+ 5-FU	18.1	5.9	=	=	=	=	=	=	↑	↑	=	=	↑	↑

Cells were exposed to different fluoropyrimidines at their IC_{50} (inhibitory concentration of 50% of the cells) for 7 days. Prior to exposure and 1, 2, 4, and 7 days after addition of the respective fluoropyrimidine, cells were processed and analysed by RT-PCR as described in "Materials and Methods." The results compare mRNA expression prior to exposure and after seven days. Signal intensities were confirmed by eye using the categories: +++ = strong signal, ++ = intermediate signal, + = weak signal. Changes in signal intensities were denoted as follows: = no change, ↑ upregulation of enzyme mRNA, ↓ downregulation of enzyme mRNA.

late synthase after exposure to 5-FU, an attenuated cytocidal effect of FdUMP was observed in the resistant cells (IC_{50} of CCL 227: 0.043 μM FdUMP; IC_{50} of CCL 227 + 5 μM 5-FU: 0.085 μM FdUMP; IC_{50} of CCL 227 + 25 μM 5-FU: 0.546 μM FdUMP). It is worth noting that this effect was reversed by modulation of FdUMP by tetrahydrofolates with remarkable sensitivity of the parent cell line and the 5-FU resistant subclones (IC_{50}:0.0015–0.005 μM FdUMP).

Similar to the experiments with naive cells, the constitutive expression of these mRNAs was also investigated in resistant subclones of CCL 227. A strong signal for thymidylate synthase mRNA was noted in the most resistant cell line, but was attenuated in the parent cell line. Thymidine kinase mRNA was present in abundance in sensitive and resistant cells. The expression of OPRT mRNA increased with the degree of resistance, whereas ribonucleotide reductase mRNA was completely omitted in the resistant clones (data not shown).

DISCUSSION

All regimens used in the treatment of metastatic colorectal cancer rely on 5-FU, although this substance has shown only limited activity when used as monotherapy. In recent years, many investigations have focused on thymidylate synthase as the target enzyme of the interaction between 5-FU and folinic acid. Considering the triple action of 5-FU on DNA, RNA and thymidylate synthase, it seems very improbable that resistance can be described with one single parameter. However, the impact of thymidylate synthase on survival of patients suffering from rectal cancer has been recently demonstrated [24, 34].

Our results illustrate that the cytotoxic pattern of fluoropyrimidines is highly individual and is characteristic for each tumour investigated *in vitro*. Because many patients with metastatic colorectal cancer will relapse within several months after an objective response by 5-FU-based chemotherapy, a model of resistance to 5-FU also has to consider the response of the pyrimidine network to cytotoxic stress. This clinical observation was reflected *in vitro* by the fast tuning of the pyrimidine network after incubation with fluoropyrimidines. Our evaluation of cytotoxicity using the metabolites of 5-FU clearly shows that anabolic activation limits the efficiency of 5-FU. The IC_{50} of 5-FU and FUrd or FdUrd often differed by more than one order of magnitude. In five cell lines, the ratio of the activity of thymidine phosphorylase and uridine phosphorylase was directly correlated to the ratio of the IC_{50} FUrd/IC_{50} FdUrd. In two other cell lines, however, we were unable to retrace these characteristic sensitivity patterns (HT 29 and CCL 247).

Antisense experiments with oligonucleotides complementary to enzyme mRNA also corroborated the importance of uridine phosphorylase and thymidine phosphorylase; the reduced translation of both enzymes abolished the cytotoxic effect of 5-FU in a dose-dependent manner, underlining the central role of the initial activation of 5-FU. Thus, recently published investigations focusing on both phosphorylases may provide evidence for their individual role in 5-FU pharmacology [12, 13].

The cytotoxicity of the fluoropyrimidines FdUrd and FdUMP was proportionally increased by modulation with tetrahydrofolates, indicating a minor importance of thymidine kinase in the activation of 5-FU. This finding is in agreement with a clinical study which proposes the combination FdUrd with leucovorin in the treatment of unresectable liver metastases in patients with primary colorectal carcinoma [35]. Ribonucleotide reductase probably influences the cytotoxicity of 5-FU by limiting the conversion of ribose to deoxyribose. The importance of ribonucleotide reductase in cellular proliferation was pointed out by the reduction of cell growth after incubation with antisense oligonucleotides. We show that a combination of 5-FU and 10 μM oligonucleotides in antisense orientation to ribonucleotide reductase mRNA results in complete cell killing. By contrast, all other antisense experiments did not reveal an influence of antisense or sense oligonucleotides alone on cellular proliferation. Thus, ribonucleotide reductase inhibitors warrant further investigation as potential therapeutic agent in combination with 5-FU.

Beyond individual characteristics of the colon tumours investigated *in vitro*, several common features were observed. The nucleosides of 5-FU had a higher cytotoxic potency than the parent compound itself, FdUrd and FdUMP being the most cytocidal of all. Because kinases are

generally abundant in tumour cells, the nucleic acids are primary targets for 5-FU. The high incorporation into RNA and not into DNA suggests efficient protective mechanisms for DNA (incorporation of 5-FU mainly <2%). Considering therapeutic long-term infusions with 5-FU, it is worth noting that after exposure even for six days with incorporation rates of 80% of the cellular uptake into RNA, a substantial part of cells recovered completely from 5-FU damage.

Because 5-FU was mainly incorporated into RNA, we tested whether functional enzymes can be translated from potentially fraudulent RNA. RT-PCR indicated intact regions of mRNA. Furthermore, intact translation was supported by high enzymatic activity of uridine phosphorylase and OPRT in resistant subclones grown in 5 μM and 25 μM 5-FU. The efficiency of 5-FU incorporation into RNA has therefore to be reconsidered and is likely of minor importance in 5-FU pharmacology, as suggested by Schmittgen and coworkers [36]. Since our data were generated from total RNA, the incorporation of 5-FU into different RNA species can be deduced from the literature only. Incorporation of 5-FU has been proven for all RNA species [1]. Although the affinity of different RNA polymerases to 5-FUTP is not known, one would expect an incorporation rate in proportion to the uracil content of the respective RNA. This would suggest that 5-FU is incorporated mainly into ribosomal RNA (80%) and into transfer RNA (10–15%). Only a minor amount of 5-FU is therefore expected in the mRNA fraction and its precursors (1–5%).

In order to understand the regulation of secondary resistance, we investigated two resistant subclones of CCL 227. The alterations observed included restrictions in the enzymatic activity of thymidine phosphorylase, but an unchanged activity of uridine phosphorylase and OPRT. At the transcriptional level, the induction of thymidylate synthase paralleled the suppression of ribonucleotide reductase in subclones resistant to 5-FU. In this context, it was surprising that FUrd could be modulated to the same extent as FdUrd. If thymidylate synthase is the major target for modulated 5-FU, two pathways can be taken into consideration: i) conversion of FUrd to 5-FU with subsequent activation via thymidine phosphorylase and/or ii) phosphorylation of FUrd to fluorouridine diphosphate with subsequent reduction via ribonucleotide reductase. Since ribonucleotide reductase is a cell cycle-dependent enzyme, it was not surprising to observe a lower mRNA expression in 5-FU-resistant subclones with a population doubling time twice that of naive cells. Given that 5-FU addresses different targets depending on the administration schedule [11], it is likely that primary and secondary resistance differ in their mechanism. Thus, induction of ribonucleotide reductase after short-term incubation may reflect a compensatory mechanism to synthesise deoxyribonucleotides for DNA repair, whereas enzyme suppression after long-term exposure may express an attenuated growth capacity overlapping a mechanism of resistance.

Short-term exposure to 5-FU acts primarily on RNA. Compatible with a high turnover of RNA in cycling cells, the cytocidal effect of 5-FU was reversible for a remarkable number of exposed cells. Enhanced expression of ribonucleotide reductase mRNA could serve the necessity of enhanced DNA repair, whose importance was stressed by substantial 5-FU incorporation after six days. In contrast, long-term exposure is characterised by a continuous influx of 5-FU and concomitant serious problems for tumour cells to maintain the integrity of the genome. Restriction of ribonucleotide reductase may reduce the conversion of FUDP to FdUDP and the subsequent incorporation into DNA. This mechanism may dominate the preference of tumour cells to divide by simply exhausting the cellular capacity to manage concomitantly both DNA repair and cell cycling under continuous exposure to 5-FU. At present, one may only speculate about the implication of ribonucleotide reductase, whose role in primary and secondary 5-FU resistance remains to be established.

In summary, colorectal tumour cells resisted 5-FU by the dynamic regulation of the pyrimidine network, adopting individual strategies in primary and secondary resistance. Bypassing the limiting steps in the activation of 5-FU is likely to circumvent resistance to 5-FU in colorectal tumour cells *in vitro*. The branching in the first anabolic step (addition of a ribose or a deoxyribose to 5-FU) is probably decisive for the mechanism of action on tumour cells. As a consequence, the first activation steps of 5-FU by the enzymes uridine phosphorylase and thymidine phosphorylase may have prognostic relevance for the cytotoxicity of 5-FU.

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