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Concentration and time dependence of the toxicity of fluorinated pyrimidines to HT 29 colorectal carcinoma cells*

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Summary. To determine the optimal concentration time factors for the fluoropyrimidines 5-fluorouracil (FU), 5-fluorouridine (FUR), and 5-fluoro-2'-deoxyuridine (FUdR) in regional chemotherapy, we tested these drugs against the colorectal carcinoma cell line HT 29 at various dosages and exposure times. The measure of cytotoxicity used was the degree of inhibition of colony formation in soft agar after drug treatment compared with untreated control cells. Colonies were visible after 6 days of growth in soft agar, so the initial evaluation of toxicity was done at this time. Additional colonies were found 10 and 16 days after the first evaluation, so the dishes containing the treated cells were also evaluated for this delayed growth phenomenon ("regrowth"), which we considered to be due to a cell growth inhibition effect of the drugs rather than a cytocidal effect. Exposure times of the cells to the drugs ranged from 5 min to 24 h and the doses, between 0.01 and 1000 μg/ml. The toxicity of FUdR was concentration-dependent, but its time dependence ceased after a relatively short exposure time. There was a cell population that was not susceptible to FUdR regardless of dose and exposure time; consequently, FUdR treatment was always accompanied by substantial regrowth of colonies. With FU and FUR, conditions could be achieved that resulted in complete cell death (no regrowth), but high concentrations and long exposure times were required with FU. With FUR, on the other hand, both cytostasis and cytoxicity could be achieved with substantially lower doses and shorter exposure times than with FU. These results indicate that FUR has the potential to be an effective drug in chemotherapy protocols not involving systemic administration.

Introduction

The fluorinated pyrimidine FU is widely used, alone or in combination with other agents, in the treatment of colorectal carcinoma, even though the response rate to this drug has usually been under 25% and the survival of patients with this particular disease has not been significantly prolonged [3, 11]. The poor clinical results with FU are most probably attributable to its relatively slow anabolism to the active metabolites in most cells. Most clinical regimens including FU have involved bolus injections which result in high peak levels of FU in the plasma, which decline rapidly with half-times of 10–15 min [2], and in vitro studies of the influence of concentration vs time (c × t) factors on the toxicity of FU against cultured human colorectal carcinoma cell lines have shown that the average exposure times attainable with bolus injections would not in most cases be likely to cause appreciable toxicity to patients' cancer cells [2, 4, 5]. It has been proposed on the basis of such studies that if FU is to have any further impact on the treatment of colon carcinoma, different treatment strategies must be evolved, aimed at improving the c × t factor of the drug [4].

We are interested in the use of regional chemotherapy as a strategy by which the c × t factor for drugs might be effectively manipulated in certain categories of patients. The rationale behind the development of regional chemotherapy is to provide a means of delivering to a specific location a higher dose of the drug for longer periods of time than is possible with systemic administration. In addition, it may be possible to use drugs in regional chemotherapy that cannot be used in systemic protocols for various reasons, such as excessive host toxicity or degradation of the drug by catabolic enzymes in the serum (e.g., thymidine phosphorylase).

In regional chemotherapy of colorectal liver metastases, the most commonly used drugs have been FU and FUdR [9, 14]. The various modes of administration of these drugs have included long-term continuous infusion via external pumps [13] or implantable pump systems [6], short-term intra-arterial infusion of ultrahigh doses of FU [1], and isolation perfusion [1, 15]. Consistently higher response rates have been achieved with hepatic arterial infusion of FU than with systemic chemotherapy [10], although correspondingly increased survival rates have not yet been reported. FUdR was thought to provide an advantage over FU because of improved liver extraction and lower systemic levels than those of FU [6], but response rates have been variable and it is still not clear whether there is any therapeutic advantage for either FU or FUdR. Part of the reason for these uncertainties may be that the treatment protocols have not been optimal with respect to drug concentration and exposure time.

In order to establish a more rational basis for the most effective use of various fluorinated pyrimidines in regional chemotherapy, we have determined concentration-vs-time

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products for the toxicity of three fluoropyrimidines, FU, FUdR, and FUR, against the HT 29 line of human colon carcinoma cells. The drug effects were determined by measuring both the permanent (cytotoxicity) and the reversible (cytostaticity) inhibition of colony-forming efficiency of the HT 29 cells in soft agar. We found that (1) FUR was the most effective of these three drugs, in terms of both cytostasis and cytotoxicity; (2) 100% cytotoxicity could be achieved with FU, but only with high doses and long exposure times; and (3) a subpopulation of cells always remained refractory to FUdR, regardless of time or dose.

Materials and methods

FUR and FUdR were generously supplied by Hoffmann-LaRoche, Basel, Switzerland. FU was purchased from Farmitalia Carlo Erba (Freiburg, FRG). The drugs were dissolved in sterile water immediately prior to use.

Test cells and media. The toxicity experiments were performed with human colorectal carcinoma cell line HT 29 (American Type Culture Collection, Rockville, Md, USA). The cells were cultured in modified PYM (Hams F12 supplemented with 8% fetal bovine serum), L-glutamine, insulin, hydrocortisone, EGF, and penicillin/streptomycin under standard conditions (5% carbon dioxide in humidified air at 37° C).

Cytotoxicity assay. Cytotoxicity assays were carried out with logarithmically growing cells using the inhibition of soft agar colony-forming efficiency as the in vitro response indicator. After harvesting with a trypsin/EDTA solution (0.25%/0.02%), the cells were tested for viability by trypan blue dye exclusion and the suspensions were adjusted to contain the same number of viable cells. The cells were exposed to the test drugs in suspension at 37° C at various exposure times and drug concentrations. At the end of the incubation period, the cells were washed twice with McCoy's 5A plus 10% fetal bovine serum using centrifugation at 200 *g*. Then the cells were seeded in a soft agar bilayer system, the base layer (feeding layer) and the top layer (cell layer) containing 0.5% and 0.3% agar (Difco), respectively. Three 35-mm dishes were prepared for each test point and the experiments performed at least twice. The cultures were observed with an inverted light microscope (Diavert, Leitz, Wetzlar). The day after seeding, the plates were checked for aggregates. Colony growth of HT 29 cells was sufficient for evaluation 6 days after seeding, reaching a plateau growth phase 16 days after seeding. The number of colonies of untreated control HT 29 cells was 1400–1600 per dish on day 6 and 2000–2400 per dish on day 16. On day 6 the colonies were counted and only colonies containing more than 30 cells were scored. Survival was determined as the percentage of colony growth in the drug-treated cells compared with colony growth in untreated controls. The cultures were reviewed 10 and 16 days later to determine colony regrowth (see Results for definition of this term).

Results

Time and concentration dependence of FU, FUR, and FUdR cytotoxicity. In order to establish c × t factors for the fluoropyrimidines FU, FUR, and FUdR for short-term as well as long-term exposures, we measured the toxicity of these drugs to HT 29 cells over a wide range of concentrations and exposure times. At concentrations ranging from 0.01 to 1000 μg/ml, all three drugs showed a clear dose-response behavior in the reduction of colony formation (Figs. 1 and 2). At the shorter incubation times, the activity of the drugs in inhibiting colony formation was in the order FUR > FUdR > FU (Fig. 1). However, with longer exposure times (1 h), the order of activity was FUR > FU > FUdR (Fig. 2). Whereas the toxicites of FU and FUR showed a considerable time dependence, changing noticeably even from a 5-min to a 20-min exposure time, the cytotoxicity of FUdR did not appear to change appreciably with time until the long-term 24-h exposure time was reached. Conditions that killed 100% of the cells were achieved with FU and FUR, but about 9% of the HT 29 cells were not susceptible to the toxic effects of FUdR, regardless of dose or exposure time.

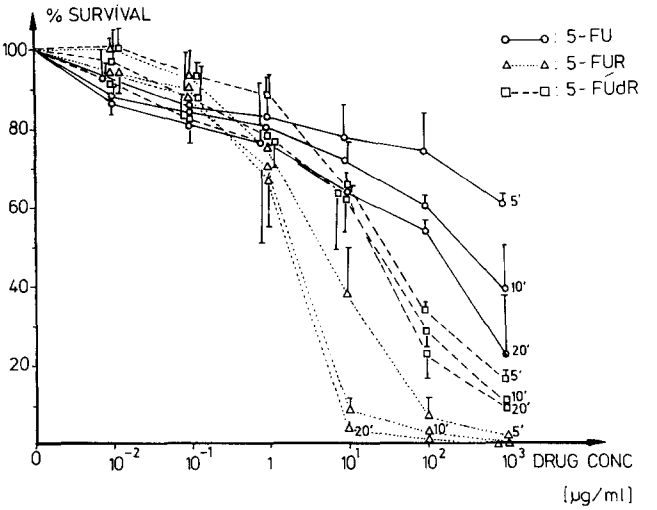

Fig. 1. The dose- and time-dependence of the toxicity of FU, FUdR, and FUR to HT 29 cells with short exposure times of 5–20 min

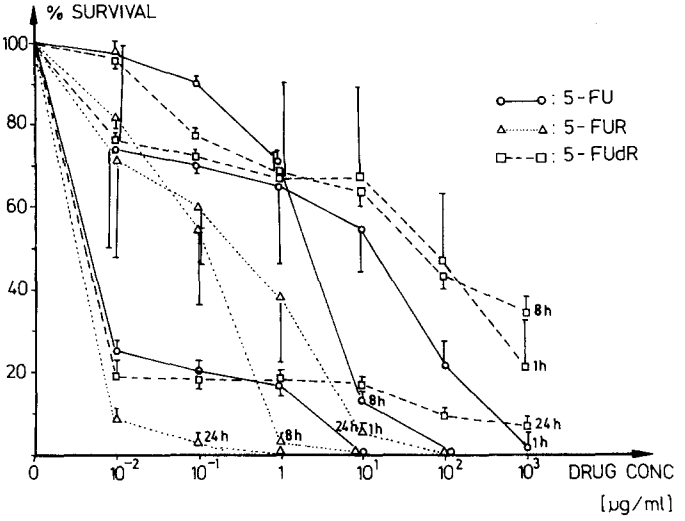

Fig. 2. The dose- and time-dependence of the toxicity of FU, FUdR, and FUR to HT 29 cells with longer exposure times of 1–24 h

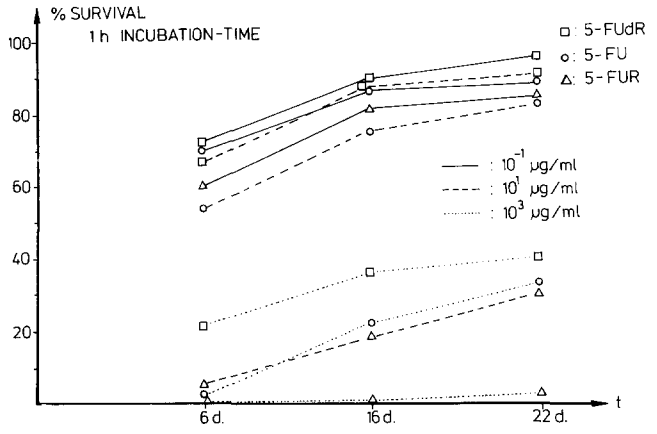

Fig. 3. Dose-dependence of the regrowth of colonies of HT 29 cells exposed to FU, FUdR, and FUR for 1 h

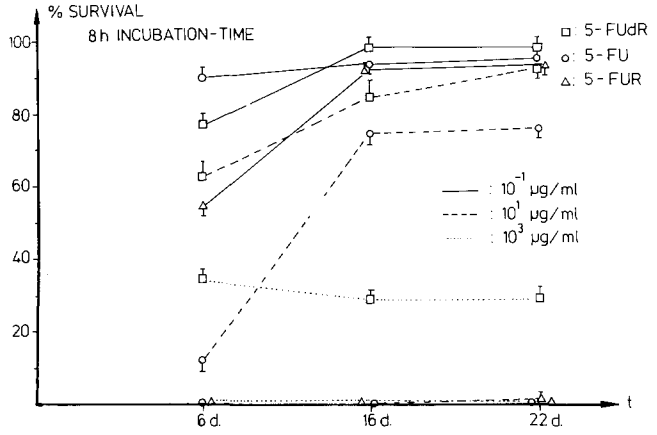

Fig. 4. Dose-dependence of the regrowth of colonies of HT 29 cells exposed to FU, FUdR, and FUR for 8 h

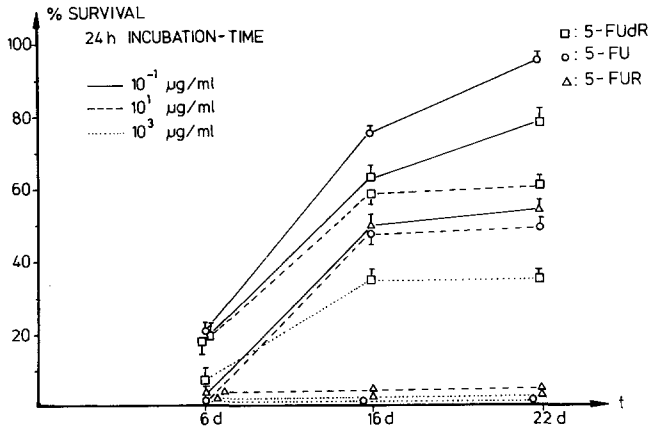

Fig. 5. Dose-dependence of the regrowth of colonies of HT 29 cells exposed to FU, FUdR, and FUR for 24 h

Delayed growth of colonies after drug treatment

We noted that after drug treatment of the cells and seeding in soft agar, more colonies were visible after 16 days of growth in the soft agar than after 6 days. We assume that this phenomenon, which we have termed "regrowth" because of its analogy to the resumption of growth of a tumor treated with drugs, is due to the presence of cells that have been damaged but not killed by the drug treatments, resulting in very slow replication of these damaged cells and thus a delay in the time required to from a visible colony. We therefore used the regrowth phenomenon as an indication of a reversible toxic (cell growth inhibition) effect of the drugs, as distinct from an irreversible cytotoxic effect causing cell death.

The regrowth potential of the cells was evaluated with various durations of exposure to each of the three fluoropyrimidines (Figs. 3–5). As might be anticipated, longer exposure times and higher drug concentrations resulted in less regrowth of the cells in all cases. With the shorter exposure of 1 h (Fig. 3) substantial regrowth was observed with FU and FUdR at all doses. Compared with the other two drugs, FUR was much more effective in preventing regrowth; with a 1-h exposure time and a concentration of 10 µg/ml there was an increase on colony formation from 5% of control to 19%, while at the high dose of 1000 µg/ml negligible regrowth was seen, indicating almost complete cytotoxicity. With longer exposure times no regrowth was observed at any concentration of FUR. With FUdR regrowth was observed at all concentrations even after a 24-h exposure time, which is consistent with the inability of this drug to inhibit colony formation completely at 6 days, even at a level of 1000 µg/ml. FU was intermediate between the last two drugs in its regrowth inhibition potency. An 8-h exposure was required before complete inhibition of regrowth was observed. However, at the lower dose of 0.1 µg/ml some regrowth was observed even after a 24-h exposure (Fig. 5).

Discussion

The purpose of this study was to determine the concentration and time dependence of the cytotoxicity and growth inhibition potency of three fluoropyrimidines, FU, FUR, and FUdR, against the HT 29 line of colon carcinoma cells. This was done over a wide range of exposure times and doses in order to cover the conditions resulting from most conceivable treatment schedules. We considered that such data could be especially useful for planning and selecting regional chemotherapy protocols, since this technique may allow more flexibility in the exposure times, doses, and types of drugs that can be used. For easier comparison of the c × t factors of these fluoropyrimidines, we have tabulated the toxicity data obtained at 6 days in Table 1.

The HT 29 cell line appears to be a reasonably good model for the response of colon tumor cells to fluoropyrimidines. A previous study showed HT 29 cells to be somewhat more sensitive to FU than were LoVo or CoLo 205 cells, but the general patterns of response of all three of these cell lines to FU were very similar [2]. Similarly, preliminary studies from the laboratory of one of the authors have shown that with 1-h exposures of LoVo cells to the fluoropyrimidines, the sequence of activity was FUR > FU > FUdR, just as with the HT 29 cells, but the LoVo cells were slightly more sensitive to FUR and slightly less sensitive to FU and FUdR than were the HT 29 cells (K. H. Link, unpublished results). The data presented in this paper, together with the above preliminary data confirming the similar responses of two cell lines to the fluoro-

Table 1. Exposure time- and concentration-dependence of the cytotoxicity of FU, FUdR, and FUR to HT 29 human colorectal carcinoma cells

Drug concentration (μg/ml)	Cell survival as percentage of that in untreated controls with exposure times indicated[a]								
	5 min			10 min			15 min		
	FU	FUdR	FUR	FU	FUdR	FUR	FU	FUdR	FUR
10^3	60± 1	16± 2	0.5± 1	39±11	11± 0	0	22±16	9.6±0	1.5± 2
10^2	74±10	33± 2	7 ± 5	60± 2	29± 5	2.4± 0	53± 3	22 ±6	1.1± 0
10	78± 9	65± 4	38 ± 5	72± 6	62±14	8.1± 3	63± 1	63 ±1	3.1± 0
1	83±10	89± 6	75 ± 8	81±11	79± 4	71 ±20	77± 4	76 ±7	66 ±27
10^{-1}	85± 8	93± 8	94 ± 8	84±11	89± 9	88 ±10	81± 8	82 ±6	91 ±12
10^{-2}	93±11	100± 5	100 ± 6	89±12	97± 9	94 ± 4	88±12	92 ±1	95 ± 5
	1 h			8 h			24 h		
	FU	FUdR	FUR	FU	FUdR	FUR	FU	FUdR	FUR
10^3	15± 3	21±18	0	0± 0	34± 3	0	0	7 ±2	0
10^2	21± 5	46±17	0	0± 0	43± 4	0	0	9.3±2	0
10	54±10	67±21	5 ± 2	13± 2	63± 3	0	0	18 ±2	0
1	65±19	69±19	38 ±17	72± 3	68± 5	3 ± 2	18± 2	18 ±2	0
10^{-1}	70±24	72±19	60 ±24	90± 2	77± 2	55 ± 2	20± 2	18 ±4	3 ± 1
10^{-2}	74±24	76±23	71 ±24	97± 2	96± 1	82 ± 3	25± 3	19 ±4	9 ± 2

[a] The cells were treated in suspension with the drugs shown as described in Materials and methods, and the number of colonies was determined after 6 days of growth in soft agar

pyrimidines, have already been used as a basis for selecting FU over FUdR in a short-term, high-dose intra-arterial chemotherapy protocol.

We found that the toxicity of the most commonly used fluoropyrimidine, FU, was highly time dependent, as other investigators have also found [2, 4, 5]. Moreover, the time required to achieve a significant cytotoxic effect was considerable. At the high concentration level of 1000 μg/ml, a 1-h exposure to FU gave only about a 96% inhibition of colony-forming efficiency after a 6-day growth assay and was followed by considerable regrowth after removal of the drug, resulting ultimately in a tumor cell kill of only about 70%. However, this combination of concentration and exposure time cannot normally be achieved in clinical practice since the normal bolus injection protocol results in a peak level of about 100 μg/ml, which persists with a half-life of about 10 min [7]. From Table 1, it is apparent that these conditions will only yield a maximum toxicity of 60%, even without taking into account regrowth stemming from the slow recovery of drug-damaged cells, which can be expected to be considerable. Thus, the generally poor response of colon tumors to the conventional FU treatment protocols is not surprising. It is clear that for FU to be an effective anticancer drug, substantial concentrations of the drug must be given for much longer exposure times than can be achieved using bolus injections.

On a biochemical level, the slow action of FU can be accounted for by its relatively sluggish rate of conversion to its corresponding ribonucleotide forms by the enzyme PRPP-phosphoribosyltransferase (Fig. 6). This is a lower-V_{max} enzyme that uses PRPP as a co-substrate. The intracellular concentrations of PRPP have often been found to be suboptimal for maintenance of maximal activity in the conversion of FU to FUMP [8, 12]. Various biochemical

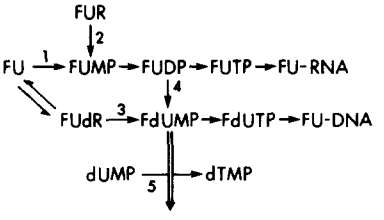

Fig. 6. Pathways of the metabolism of the fluoropyrimidines. FUMP, FUDP, FUTP are 5-fluorouridine-5'-mono-, di-, and triphosphates, respectively; FdUMP and FdUTP are 5-fluoro-2'-deoxyuridine-5'-mono- and triphosphates, respectively. Enzymes are: *1*, pyrimidine-PRPP phosphoribosyltransferase; *2*, uridine kinase; *3*, thymidine kinase; *4*, ribonucleotide reductase; *5*, thymidylate synthetase; *6*, thymidine phosphorylase

modulation protocols are currently being pursued in an effort to raise intracellular PRPP levels, with the aim of accelerating this conversion and thereby increasing the c × t product for FU without the necessity for longer exposure times [8].

The deoxyribonucleoside FUdR is apparently often regarded as another from of FU, since the two drugs appear to be used interchangeably in clinical protocols. However, there is considerable evidence that FU has multiple mechanisms of action, including inhibition of thymidylate synthetase and incorporation into RNA, whereas the activity of FUdR is quite specifically directed at the inhibition of thymidylate synthetase because of its rapid conversion to FdUMP by thymidine kinase (Fig. 6) [8]. In most cell culture systems FUdR is not appreciably cleaved to FU by degradative enzymes, although this may not be the case in vivo [8]. Furthermore, FU and FUR are not phase-specific,

and they exert toxicity in all phases of the cell cycle, presumably because of their RNA-directed mechanism of action, whereas FUdR is an S-phase-specific drug [8]. The FUdR-resistant subpopulation of HT 29 cells most probably consists of cells that are not in S-phase, and therefore not susceptible to the effects of FUdR, at the time of treatment. Because of this effect, it can be predicted that the antitumor efficacy of FUdR will be limited unless ways can be found to recruit the tumor cells into a proliferative state so that more of them are rendered susceptible to the drug. In addition to this problem, we found that regardless of the exposure time and concentration, there was always a high rate of regrowth of cells treated with FUdR, indicating that the mode of action of the drug is largely cytostatic.

FUR was by far the most effective fluoropyrimidine, in terms of both the cytostasis and the cytotoxicity achieved in the shortest exposure time. The superiority of FUR to the other drugs can be accounted for by two factors: (1) the rapid cellular uptake and efficient conversion of FUR to nucleotides compared with FU [16], and (2) the irreversible and non-phase -specific RNA-directed cytotoxic effects of the drug. These results suggest that it might be worthwhile reevaluating the clinical use of FUR for certain types of regimens prescribing nonsystemic use.

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