0026-895X/86/060637-06$02.00/0

MOLECULAR PHARMACOLOGY, 29:637-642

Precursor-Dependent Differences in the Incorporation of Fluorouracil in RNA

CHRIS H. TAKIMOTO,[1] EDWIN C. CADMAN,[2] and R. DOUGLAS ARMSTRONG[3]

Cancer Research Institute (C.H.T., E.C.C., R.D.A.) and Department of Pharmacology (R.D.A.), University of California, San Francisco, California 94143 and Department of Pharmacology (C.H.T.), Yale University School of Medicine, New Haven, Connecticut 06510

Received June 26, 1985; Accepted February 25, 1986

SUMMARY

The distribution and level of 5-fluorouracil (FUra) incorporation into RNA were studied in S-180 murine tumor cells for two different fluoropyrimidine nucleosides: 5-fluorouridine (FUrd) and 5′-deoxy-5-fluorouridine (5′-dFUrd) under both cytotoxic (thymidine nonreversible) and nontoxic conditions. Exposure of cells to 200 μM 5′-dFUrd for 6 hr resulted in minor thymidine-nonreversible toxicity (11% cell kill) and produced an FUra incorporation level of 12.5 pmol of FUra/μg of RNA. Exposure of cells to 1 μM FUrd for 6 hr resulted in considerably more toxicity (74% cell kill) but was found to produce less FUra incorporation (7.8 pmol of FUra/μg of RNA) than the nontoxic concentration of 5′-dFUrd. Subcellular fractionation (i.e., nucleolar, nucleoplasmic, cytoplasmic) was completed, and the density of FUra incorporation from 5′-dFUrd was found to be approximately equally distributed between nucleoplasmic RNA and nucleolar RNA, independent of toxicity or concentration levels (average nucleolar/nucleoplasmic FUra ratio = 0.75). FUrd, however, was preferentially incorporated into nucleolar RNA (average nucleolar/nucleoplasmic FUra ratio = 22). The nontoxic accumulation of FUra in RNA of cells treated with 200 μM 5′-dFUrd was also associated with the mature cytoplasmic 28 S and 18 S rRNA, the levels of which increased considerably with exposure time. In contrast, in cells exposed to 1 μM FUrd, there was very little FUra measured in the mature 28 S and 18 S rRNA, consistent with an inhibition of rRNA processing. It is concluded that 5′-dFUrd can produce large nontoxic levels of FUra accumulation in cellular RNA compared with FUrd because total FUra incorporation in nuclear RNA was almost equally dispersed between nucleoplasmic and nucleolar RNA, whereas nuclear FUra incorporation from FUrd was preferential for nucleolar RNA. Also, for nontoxic doses of 5′-dFUrd, unaltered rRNA processing over time can result in the large accumulation of FUra-containing rRNA in the cytoplasm.

The fluoropyrimidines are commonly used in the treatment of human cancer, although their precise mechanism of cytotoxicity is still not completely understood. Most studies on the mechanism of action of the fluoropyrimidines have focused on the generation of fluorodeoxyuridine monophosphate which can inhibit the enzyme thymidylate synthetase and thereby suppress DNA synthesis (1). Clearly, in cells unable to overcome this block, the FdUMP-mediated suppression of thymidylate production is a potent cytotoxic mechanism. However, other evidence suggests that the antitumor activity of the fluoropyrimidines may also be related to the formation of fluororibonucleotides (e.g., FUTP) and the subsequent incorporation of FUra into RNA. Under certain conditions, the toxicity of FUra correlates with its incorporation into RNA and not with the inhibition of DNA synthesis (2–4). Also, rescue studies showed that the addition of dThd can prevent the block in DNA synthesis caused by FUra, but it cannot always prevent cell death, particularly at high FUra concentrations (5). This lethality which is not reversed by dThd has been referred to as the RNA-mediated cytotoxicity of the fluoropyrimidines. Each of the fluoropyrimidines has a different ability to produce this toxicity. The nucleoside, FUrd, is a very active agent in producing dThd-nonreversible cytotoxicity, whereas the nucleoside, 5′-dFUrd, is relatively nonpotent (3, 6). In addition, FUra activity can be increased by combining the drug with biochemical modulators such as methotrexate, where it was shown that this increased toxicity correlated with the incorporation of FUra into RNA and not with an increased

This study was supported by Grants CA 363704 and CA 36552 from the National Cancer Institute; Grants CH-145 and CH-239 from the American Cancer Society; and grants from the Cancer Research Coordinating Committee and the Research Evaluation and Allocation Committee (MSC 23), University of California.

[1] This work was completed by C. H. T. as part of the requirements for a doctoral thesis in the Medical Scientist Training Program and the Department of Pharmacology, Yale University School of Medicine.

[2] E. C. C. was a recipient of a Faculty Research Award from the American Cancer Society.

[3] R. D. A. was supported by a Leukemia Society of America Award and by National Institutes of Health Fellowship Award CA 07162.

ABBREVIATIONS: FUra, 5-fluorouracil; 5′-dFUrd, 5′-deoxy-5-fluorouridine; FUrd, 5-fluorouridine; FUTP, 5-flurouridine triphosphate; dThd, thymidine; HPLC, high performance liquid chromatography; PBS, phosphate-buffered saline; SDS, sodium dodecyl sulfate; VRC, vanadyl-ribonucleotide complex.

drug effect on DNA synthesis (7). Finally, fluoropyrimidine-induced alterations in RNA metabolism have been shown to occur, but a precise mechanism that can explain dThd-nonreversible cytotoxicity has not been clearly identified. Currently, the best characterized action of the fluoropyrimidines on RNA is the inhibition of rRNA processing, the relevance of which was first studied in mammalian cells by Wilkinson and Pitot (8), and more recently by Herrick and Kufe (9). In total, these observations have led to a renewed interest in the incorporation of FUra into RNA and its possible relationship to fluoropyrimidine cytotoxicity.

Other investigators have had difficulty correlating tumor response with fluoropyrimidine incorporation into RNA. Houghton and Houghton (10) examined the effect of equally toxic doses of three fluoropyrimidines, FUra, FUrd, and fluorodeoxyuridine on human colonic tumor xenografts in nude mice. The activity of these different drugs failed to correspond with the level of FUra incorporation into total cell RNA, although dThd-nonreversible toxicity was not selectively examined. We have also observed that the absolute accumulation of FUra into total cell RNA does not always correlate with dThd-nonreversible cytotoxicity (11). Two of the fluoropyrimidines, FUrd and 5′-dFUrd were both effective substrates for FUra incorporation into RNA. However, the resulting dThd-nonreversible cytotoxicity was very different, with FUrd showing much more potency than 5′-dFUrd at doses that produced equal levels of FUra-containing RNA. Thus, although both drugs produced high levels of incorporation into RNA, they differed in their overall impact on RNA metabolism. This suggests that these two precursors of FUTP may result in incorporation into different types or classes of RNA which may lead to different levels of cytotoxicity. The present study was initiated to compare the RNA resulting from these two treatments to better characterize any difference that these two fluoropyrimidines have on RNA metabolism and, therefore, dThd-nonreversible cytotoxicity.

Materials and Methods

Chemicals. 5′-dFUrd was obtained from Hoffmann LaRoche, Inc. (Nutley, NJ). FUrd was purchased from Calbiochem-Behring Corp. (La Jolla, CA). [6-^{3}H]-5′-dFUrd (16 Ci/mmol), [6-^{3}H]-FUrd (18 Ci/mmol), and [5-^{3}H]cytidine (22 Ci/mmol) were purchased from Moravek Biochemicals (Brea, CA). dThd and all other biochemicals, unless otherwise designated, were purchased from Sigma Chemical Co. (St. Louis, MO). [^{3}H]-5′-dFUrd was purified by HPLC prior to its use in these studies. [^{3}H]FUrd was checked for purity by HPLC analysis, and 98% of the radiolabel was found to be associated with FUrd, about 0.5% with FUra and 1.5% as H_2O. Both HPLC procedures were completed as described below.

Cell culture. Murine Sarcoma-180 (S-180) cells were maintained at 37° in a 5% CO_2 atmosphere as suspension cultures in Roswell Park Memorial Institute Medium 1640 supplemented with 10% heat-inactivated horse serum plus streptomycin (100 μg/ml) and penicillin (100 units/ml). Tissue culture supplies were all purchased from GIBCO (Grand Island, NY).

Clonogenic survival. Exponentially growing cells were exposed to various concentrations of fluoropyrimidines for 6 hr. After drug treatment, cells were washed once with sterile phosphate-buffered saline (PBS: 100 mg of $CaCl_2$, 200 mg of KH_2PO_4, 59 mg of $MgSO_4$, 800 mg of NaCl, and 1150 mg of $NaHPO_4$ in a 1-liter sterile aqueous solution) and counted in a model ZB1 Coulter counter (Hialeah, FL). dThd at 10 μM was added to the cloning media following removal of the drug to circumvent any inhibition of thymidylate synthetase. Drug toxicity was assessed using a soft agar cloning procedure described previously (9). Viability was defined as the ability of a cell to produce progeny which are visible as distinct individual colonies or clones. Percentage of clonal growth was determined by the ratio of clonal growth of treated cells compared to control cells × 100. Cloning efficiency of untreated cells averaged 87%, with 300 cells plated per condition.

Isolation of total cell RNA. Cells were washed once with ice-cold PBS and suspended in iced lysis buffer consisting of 1.7% SDS, 0.8% Triton X-100, 25 mM Tris-HCl (pH 7.0), 8.5 mM EDTA, 8.3 mM NaCl, 0.8% ethanol, and 50 μM vanadyl-ribonucleotide complex (VRC, Bethesda Research Laboratories, Rockville, MD). The mixture was vortexed vigorously and stored on ice for 10 min. The nuclei were disrupted and the DNA was sheared by repeated squirting of the viscous solution through a sterile 19 gauge needle. The sample was extracted twice with an equal volume of phenol/chloroform (1:1) and twice with an equal volume of chloroform/isoamyl alcohol (24:1). Three volumes of absolute ethanol and 1/20 volume of 5 M NaCl were added and the nucleic acids were precipitated overnight at −20°. The precipitated RNA and DNA were spun down at 5000 × *g* for 15 min and resuspended in 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, and 50 μM VRC. RNase-free DNase I, purchased from Worthington (Malvern, PA) was added to a final concentration of 50 μg/ml and the solution was incubated at 37° for 30 min. The solution was made with 10 mM EDTA and 0.2% SDS, and the purified RNA was extracted three times with penol/chloroform as before and precipitated in ethanol.

Identification of ^{3}H in RNA. Confirmation that the ^{3}H incorporation into RNA resulting from exposure of cells to [^{3}H]-5′-dFUrd or [^{3}H]FUrd was only associated with [^{3}H]FUra base residues was completed as follows. Following isolation as described above, 2 μg of RNA were solubilized in a buffer of 20 mM sodium succinate and 5 mM $CaCl_2$ (pH 6.0). Micrococcal nuclease 92 μg/μg of RNA), spleen phosphodiesterase (2 μg/μg of RNA), an acid phosphatase (1 unit/μg of RNA) were added and the solution was incubated at 37° for 1.5 hr. The reaction was stopped and enzymes were precipitated by adjusting the assay mixture to 10% trichloroacetic acid. The acid-soluble fraction was neutralized and analyzed for nucleosides by HPLC. The HPLC procedure was completed using a 5-μ RP-18 Ultrasphere-ODS column (4.6 × 25 cm) with a 0.1 mM $NaPO_4$ (pH 6.0) running buffer at a flow rate of 1 ml/min. FUrd eluted at 19.5 min using this procedure.

Isolation of cytoplasmic nucleolar, and nucleoplasmic RNA. The subcellular fractionation of total cell RNA was adopted from a method developed by Penman *et al.* (13). Cells were washed once with ice-cold PBS and suspended in lysis buffer A which consisted of 10 mM Tris-HCl (pH 7.0), 10 mM NaCl, 1% Triton X-100, 1% ethanol, and 50 μM VRC. This mixture was vortexed and stored on ice for 10 min. Nuclei were collected by spinning at 700 × *g* for 10 min, and the pellet was washed once with lysis buffer A. The supernatants were combined to form the cytoplasmic RNA fraction. The nuclear pellet was suspended in 2 ml of a DNase buffer containing 0.5 M NaCl, 0.05 M $MgCl_2$, 0.01 M Tris-HCl (pH 7.4), and 200 μg of DNase I; then, this solution was incubated at 37° for 3–5 min until the pellet dissolved. Nucleoli were then pelleted by spinning at 10,000 × *g* for 5 min. The supernatant was collected as the nucleoplasmic RNA fraction. The pellet containing nucleolar RNA was resuspended in 10 mM EDTA and 0.2% SDS, and all three fractions (cytoplasmic, nucleolar, and nucleoplasmic RNA) were extracted with phenol/chloroform and precipitated in ethanol.

FUra-RNA determination. Tritium-labeled fluoropyrimidines were incubated with S-180 cells for 6 hr. Different species of RNA were isolated as described above. The purified RNA was dissolved in distilled water and quantitated by measuring the absorbance at 260 nm (1 A_{260} unit = 40 μg of RNA). Radioactivity in the sample was assayed by liquid scintillation counting. Specific activities for [^{3}H]-5′-dFUrd ranged from 1 to 50 Ci/mol and [^{3}H]FUrd ranged from 50 to 100 Ci/mol.

Electrophoresis of RNA. RNA in the 4 S to 50 S range was fractionated on vertical 1.5% agarose slab gels containing 6 M urea and 0.015 M iodoacetate with a Tris-phosphate running buffer (0.04 M Tris,

0.036 M NaH_2PO_4, 0.001 M EDTA, pH 7.4). RNA bands were visualized under UV light after staining with ethidium bromide. Incorporation of radiolabeled fluoropyrimidines into the different size classes of RNA was determined by slicing the RNA bands out of the gel and incubating the slices in 0.1 N NaOH overnight to hydrolyze the RNA. The gel slices were neutralized with HCl and the radioactivity was assayed by liquid scintillation counting.

Results

Fluoropyrimidine cytotoxicity in S-180 cells. The effect of a 6-hr treatment of S-180 cells with 5′-dFUrd and FUrd was determined by cloning the drug-treated cells in soft agar in the presence and absence of 10 μM dThd (Fig. 1). The addition of dThd was able to reduce but not eliminate cytotoxicity for both drugs and it did not affect clonal growth by itself. The approximate LD_{50} values for a 6-hr exposure in the absence of dThd were 13 μM for 5′-dFUrd and 0.04 μM for FUrd. When cells were cloned in the presence of 10 μM dThd, the LD_{50} values increased to 540 μM for 5′-dFUrd and 0.62 μM for FUrd. These results demonstrate that FUrd was approximately 1000-fold more potent than 5′-dFUrd in producing dThd-nonreversible cytotoxicity.

The cytotoxicity expressed in the presence of 10 μM dThd appears unrelated to any acute suppression of DNA synthesis. Following the completion of the fluoropyrimidine exposure, cells were pulsed for 0.5 hr with [^{3}H]deoxyguanosine, and the level of incorporation into DNA was used as a measure of DNA synthesis. As shown in Table 1, dThd did reverse the suppression of DNA synthesis caused by either FUrd or 5′-dFUrd. However, as was observed in Fig. 1, cytotoxicity was not always

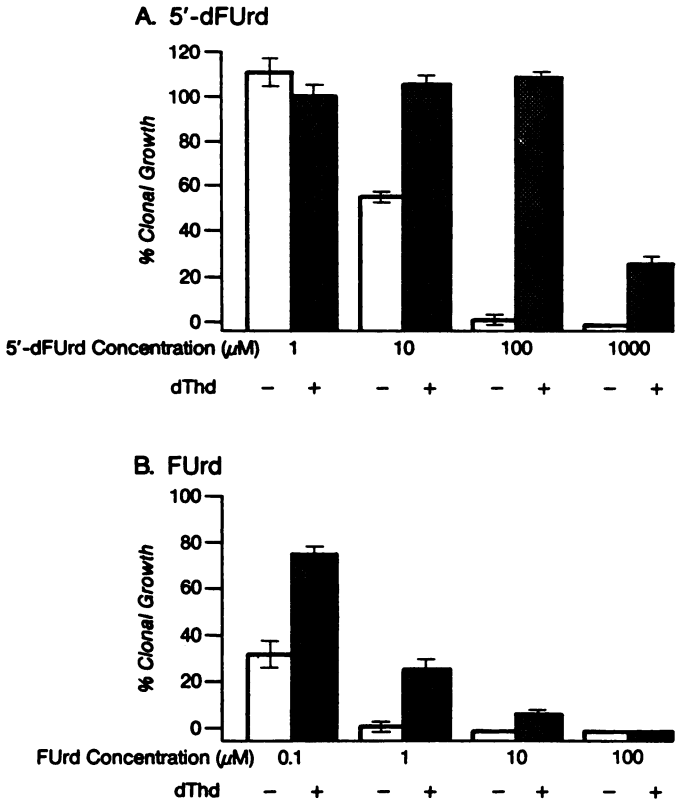

Fig. 1. Clonal growth of fluoropyrimidine-treated S-180 cells. Cells were treated for 6 hr with different concentrations of 5′-dFUrd (A) or FUrd (B) and then cloned in the presence or absence of 10 μM dThd as described under Materials and Methods. *Bars* are means ± standard error (n = 3).

TABLE 1

Effect of dThd on DNA synthesis

S-180 cells were exposed to FUrd or 5′-dFUrd for 4 hr, and 10 μM dThd was added for the next 2 hr. These cells, and cells in which dThd was not added, were then washed and resuspended in drug-free medium. At 0 hr and 18 hr following the drug washout, cells were pulsed for 0.5 hr with [^{3}H]deoxyguanosine and its incorporation into DNA was quantitated.

Sample	% DNA Synthesis[a]			
	0 hr		18 hr	
	−dThd	+dThd	−dThd	+dThd
Control	100 ± 7	100 ± 9	100 ± 8	100 ± 5
0.1 μM FUrd	45 ± 6	91 ± 6	18 ± 6	54 ± 7
1.0 μM FUrd	26 ± 6	86 ± 8	9 ± 3	51 ± 3
5.0 μM FUrd	27 ± 7	80 ± 3	7 ± 5	8 ± 4
10 μM FUrd	22 ± 4	71 ± 14	8 ± 6	20 ± 4
50 μM 5′-dFUrd	39 ± 6	93 ± 4	17 ± 2	98 ± 4
100 μM 5′-dFUrd	33 ± 3	91 ± 6	8 ± 5	93 ± 2
200 μM 5′-dFUrd	24 ± 8	84 ± 5	8 ± 3	86 ± 5
1000 μM 5′-dFUrd	19 ± 4	76 ± 2	6 ± 2	68 ± 6

[a] n = 3. Values are means ± SD.

TABLE 2

FUra incorporation into S-180 RNA

S-180 cells were incubated for 6 hr with different concentrations of [^{3}H–5′-dFUrd or [^{3}H]FUrd. RNA fractions were isolated and the FUra incorporation was determined as described under Materials and Methods.

Treatment	FUra incorporation into RNA				
	Total Cell	Cytoplasmic	Nucleolar	Nucleoplasmic	Ratio[a]
	pmol of FUra/μg of RNA				
5′-dFUrd					
50 μM[b]	5.3	4.3	7.4	8.4	0.88
200 μM[c]	11.9 ± 2.2	7.9 ± 1.1	18.1 ± 1.4	20.1 ± 1.4	0.91
1000 μM[b]	64.7	45.8	76.5	174	0.44
FUrd					
0.1 μM[b]	0.5	0.4	0.9	0.4	2.25
1 μM[c]	7.8 ± 2.1	7.3 ± 0.7	24.3 ± 8.3	1.2 ± 0.8	20.2
10 μM[b]	87.8	88.0	128	3.7	34.7

[a] Ratio of nucleolar FUra incorporation to nucleoplasmic FUra incorporation.
[b] Values represent the average of two separate experiments.
[c] Values represent the mean of four separate experiments ± SD.

reversed proportionately. These results suggest that the dThd-nonreversible cytotoxicity is unrelated to an acute suppression of DNA synthesis.

FUra incorporation into RNA. Following a 6-hr exposure to nontoxic and toxic concentrations of fluoropyrimidines, the total cell RNA was isolated and the FUra incorporation was determined (Table 2). Both fluoropyrimidines were effective precursors for FUra incorporation into RNA; however, much higher concentrations of 5′-dFUrd were needed to produce the same level of FUra in RNA as FUrd. Subcellular fractionation of S-180 cells allowed for the isolation of cytoplasmic, nucleolar, and nucleoplasmic RNA (13). The distribution pattern of FUra in each of these subcellular fractions was very different for the two fluoropyrimidines (Table 2). In cells exposed to FUrd, the highest density of FUra incorporation (measured as pmol of FUra/μg of RNA) was found in the nucleolar fraction, whereas the lowest density was found in the nucleoplasmic RNA fraction. This was true regardless of the concentration or toxicity level of FUrd, as the average nucleolar/nucleoplasmic ratio was 22. In marked contrast, 5′-dFUrd produced a large density of FUra incorporation in the nucleoplasmic RNA, relative to what was observed for FUrd. For 5′-dFUrd, the nucleolar/nucleoplasmic ratio was always less than 1, with an average of 0.75. This result was obtained independently of the level of cytotox-

icity produced. Thus, the relative pattern of FUra distribution in the nucleoplasmic RNA varied dramatically with the precursor used, and this pattern was consistent regardless of the concentration of drug employed. Additional experiments confirmed that the total amount of RNA isolated per cell was unchanged for either drug in all of these experimental conditions. To confirm that the radiolabel measured in the RNA was associated with FUra-base residues, RNA was enzymatically hydrolyzed and dephosphorylated to nucleosides, which were then characterized by HPLC (see Materials and Methods). Greater than 97% of all radioactivity associated with nucleosides was found to be [^{3}H]FUrd from both cells treated with [^{3}H]5′-dFUrd and [^{3}H]FUrd.

Comparison of FUra-labeled RNA with dThd-nonreversible cytotoxicity. With time, the treatment of S-180 cells with 200 μM 5′-dFUrd and 1 μM FUrd produced roughly similar levels of total cell FUra in RNA (Table 3). At 1 hr, the level of FUra in RNA was lower in 5′-dFUrd-treated cells; however, by 6 hr, this level was slightly higher than that seen in cells treated with 1 μM FUrd. Despite this similar degree of incorporation, the dThd-nonreversible cytotoxicities of these two exposures differed greatly (Table 3). The 6-hr treatment with 5′-dFUrd was relatively nontoxic, resulting in a clonal growth of 89% of the control when supplemented with dThd. The 6-hr treatment with FUrd was more toxic, showing 26% clonal growth in the presence of dThd. Thus, despite the fact that 5′-dFUrd generated greater levels of FUra in total cell RNA (12.5 and 7.8 pmol/μg, respectively, for 200 μM 5′-dFUrd and 1 μM FUrd), it was markedly less cytotoxic. The comparatively low level of FUra incorporation from 5′-dFUrd at 1 hr would suggest that the molar rate of incorporation per unit time was lower for 5′-dFUrd, as has been reported previously (14).

FUra incorporation in RNA fractionated by electrophoresis. Gel electrophoresis was used to examine the RNA from cells exposed to concentrations of 5′-dFUrd and FUrd which generated similar levels of FUra in total cell RNA. S-180 cells were exposed to 200 μM [^{3}H]-5′-dFUrd or 1 μM [^{3}H] FUrd for 6 hr and the total cell RNA was isolated. Electrophoresis (Fig. 2) of the RNA on 1.5% agarose/6 M urea gels allowed for the separation of the precursor rRNAs (45 S, 36 S, and 32 S), the mature rRNAs (28 S, and 18 S), and the small molecular weight RNAs (4 S to 8 S). Substantial FUra incorporation occurred in each of these species of RNA following treatment with either drug. The respective incorporation of FUra in RNA in cells treated for 1, 3, and 6 hr with 200 μM 5′-dFUrd was compared with the FUra pattern in the RNA from cells treated for 6 hr with FUrd (Fig. 3). The predominant differences between the two treatments were noted in the rRNA. Treatment with 5′-dFUrd resulted in a time-dependent increase in FUra accumulation in the mature 28 S and 18 S rRNA fractions, with 39% of the total FUra incorporation found in these species at 6 hr. In contrast, FUrd caused a greater accumulation in the 45 S, 36 S, and 32 S precursor rRNA and showed relatively little incorporation into the 28 S and 18 S mature rRNA. Only 10% of the total FUra in RNA was found in the mature 28 S and 18 S rRNA following a 6-hr exposure to 1 μM FUrd. These results are consistent with the FUrd producing a block in the processing of rRNA to the mature species (5). However, the less toxic 5′-dFUrd did not appear to shut down rRNA processing since, with an increase in time, there was a proportionally greater amount of radiolabeled drug accumulating in the mature 28 S and 18 S rRNA. No evidence for a time-dependent impairment in the production of tRNA was observed for either drug treatment. Thus, despite producing equal levels of FUra in total cell RNA, these concentrations of 5′-dFUrd and FUrd distributed differently in the distinct types of RNA

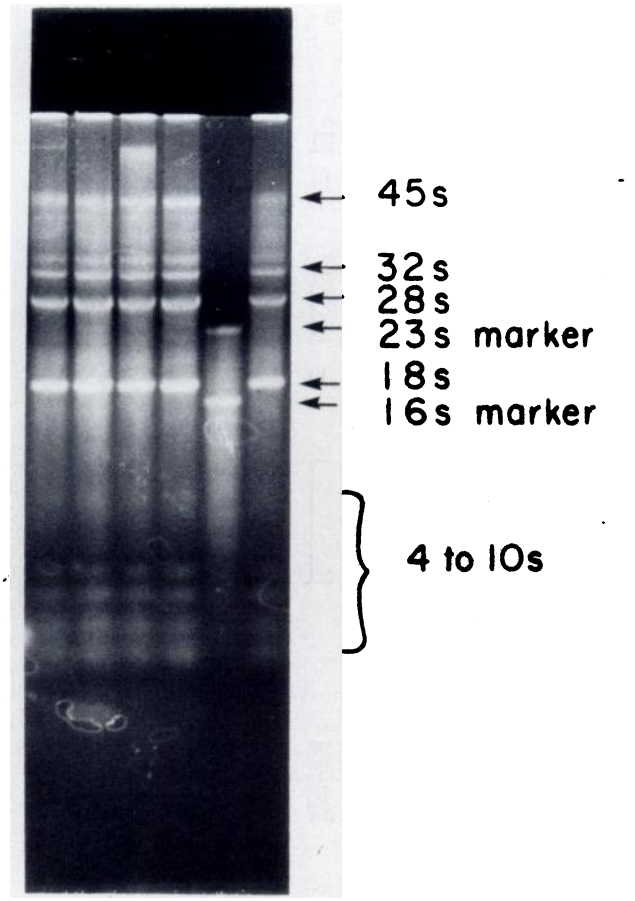

Fig. 2. Agarose/urea gel electrophoresis of S-180 total cell RNA. S-180 cells were incubated with [^{3}H]5′-dFUrd or [^{3}H]FUrd for 1.3 or 6 hr. The RNA was isolated and one A_{260} unit was electrophoresed as described under Materials and Methods. This allows for the resolution of the precursor rRNAs (45 S, 36 S, and 32 S), the mature rRNAs (28 S and 18 S), and the transfer and small molecular weight RNAs (8 S to 4 S). From *left* to *right*: lane 1 = 200 μM 5′-dFUrd, 1 hr; lane 2 = 200 μM 5′-dFUrd, 3 hr; lane 3 = 200 μM 5′-dFUrd, 6 hr; lane 4 = 1.0 μM FUrd, 6 hr; lane 5 = 16 S and 23 S rRNA marker; lane 6 = control.

TABLE 3

Clonal growth of S-180 cells and FUra incorporation into total cell RNA

S-180 cells were incubated for 6 hr with [^{3}H]5′-dFUrd or [^{3}H]FUrd. A fraction of the cells was then cloned in soft agar in the presence of 10 μM dThd and the remaining cells were used to determine incorporation into total cell RNA as described under Materials and Methods.

Treatment	FUra incorporation in total cell RNA[a]			Clonal growth[a,b]
	1 hr	3 hr	6 hr	
	pmol of FUra/μg of RNA			*% of control*
200 μM 5′-dFUrd	2.0 ± 0.7	7.3 ± 0.9	12.5 ± 2.3	89 ± 9
1 μM FUrd	3.2 ± 0.5	5.5 ± 1.2	7.8 ± 2.5	26 ± 7

[a] n = 3. Values are means ± SD.
[b] Following 6 hr exposure.

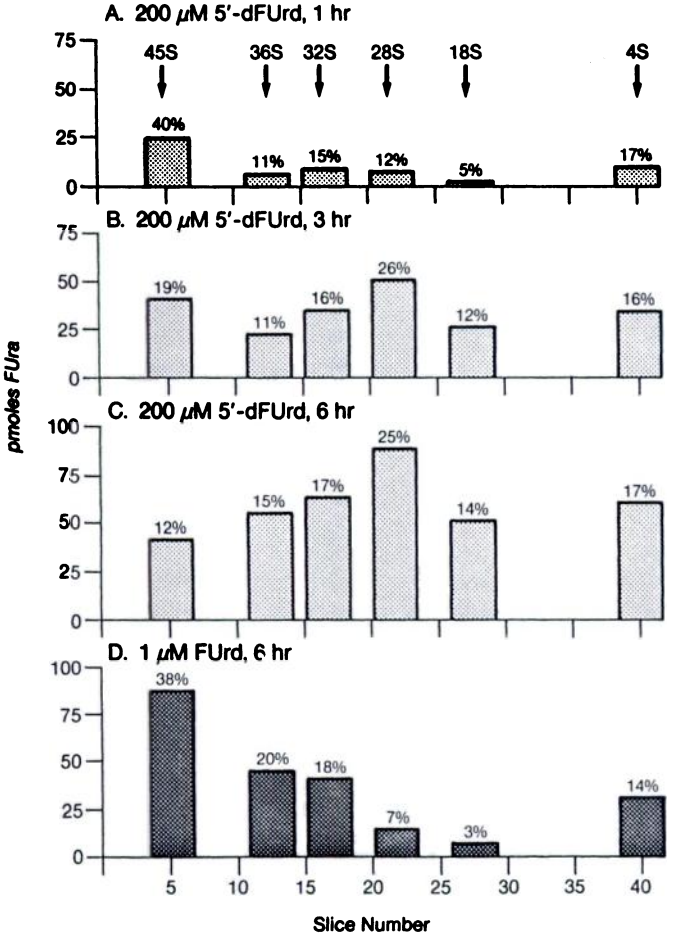

Fig. 3. FUra incorporation into specific RNA species. S-180 cells were incubated for different times with 200 μM [^{3}H]-5'-dFUrd or 1 μM [^{3}H] FUrd. The total cell RNA was isolated and one A_{260} unit of RNA was electrophoresed as shown in Fig. 2. The bands were then sliced out of the gel and the picomoles of FUra contained in each were determined as described under Materials and Methods. The percentage values indicate the fraction of total FUra incorporation occurring in that RNA band. The total picomoles of FUra quantitated in each lane were: (A) 65 pmol, (B) 212 pmol, (C) 372 pmol, and (D) 232 pmol. These results are from one experiment the patterns of which were reproduced identically in two additional comparative experiments.

separated by gel electrophoresis. These differences appeared to be related to a differential effect of these two drugs on rRNA processing.

Discussion

This study compared the two fluoropyrimidine nucleosides FUrd and 5'-dFUrd, which are both precursors for FUra incorporation into RNA. Previous studies with these two compounds demonstrated that both are converted to FUTP, although they differ in their enzymatic pathways for activation and in their relative rates of metabolism (15). In addition, their dThd-nonreversible cytotoxicities also differ, with FUrd being much more potent than 5'-dFUrd, despite the fact that both can produce similar amounts of FUra incorporation into total cell RNA (11). This suggested that the FUra incorporation from the more toxic FUrd is different from that which occurs from the less toxic 5'-dFUrd treatment. We initiated the present study to confirm the above observation and to examine the RNA incorporation resulting from these two drug treatments in order to characterize the precursor-dependent differences in the incorporation of FUra into RNA.

Both drugs were cytotoxic to S-180 cells, although in the presence of exogenous dThd, FUrd was approximately 1000-fold more potent than 5'-dFUrd. Each agent also effectively incorporated FUra into S-180 RNA, but subcellular fractionation revealed differences in their patterns of distribution in the various RNA compartments. The nucleoplasmic RNA fraction, which reportedly (13) contains heterogeneous nuclear or pre-mRNA, showed the greatest difference between the two drugs. For all concentrations of FUrd examined, the level of FUra in nucleoplasmic RNA was consistently much lower than in the nucleolar or cytoplasmic RNA fractions (Table 2). 5'-dFUrd, however, uniformly produced approximately equal levels of FUra incorporation between the nucleoplasmic and nucleolar RNA. These results were true regardless of whether nontoxic or toxic concentrations were used. The differences between the two treatments in the labeling of cytoplasmic and nucleolar RNA were not as dramatic. Therefore, the incorporation of FUra in the nucleoplasmic RNA, relative to the other RNA fractions, appears to be highly dependent upon the precursor used and is not related to concentration or toxicity levels.

A potential explanation for this apparent differential distribution of FUra into nuclear RNA is the compartmentalization of ribonucleotide precursor pools. Genchev *et al.* (16) have previously reported that nuclear pyrimidine ribonucleotide pools for RNA biosynthesis may be compartmentalized. Different precursors of UTP were found to result in incorporation into different species of nucleolar RNA: [^{3}H]uridine preferentially labeled pre-rRNA, whereas [^{3}H]orotate, which is also a precursor of UTP, preferentially labeled the heterogeneous nuclear RNA or pre-mRNA. Shani and Danenberg (17) have extended these observations somewhat to the fluoropyrimidines. They found evidence for distinct, non-equilibrating precursor pools of FUTP for the synthesis of various species of RNA which were fed by different metabolic pathways. FUra and FUrd were metabolized in such a way that each was channeled into different ribonucleotide compartments and ultimately into different classes of RNA. Thus, two different fluoropyrimidines which produce similar levels of incorporation into total cell RNA may actually be found in different types of RNA.

With increasing periods of exposure, it was apparent that another factor could markedly contribute to the differential FUra accumulation in RNA seen with these two agents. In cells treated with 200 μM 5'-dFUrd, there was a time-dependent increase in the accumulation of FUra in the mature cytoplasmic 28 S and 18 S rRNA, with a large fraction of the total cell FUra incorporation occurring in these rRNA by 6 hr (Fig. 3). This was in marked contrast to 1 μM FUrd, where very low levels of FUra incorporation were found in rRNA at 6 hr, a pattern consistent with an FUrd-induced block in rRNA processing (8). In the case of 5'-dFUrd, it was clear that with time the unaltered production of rRNA resulted in the large accumulation of FUra in total cell RNA and partially explains why a 6-hr exposure to 5'-dFUrd can have greater, yet nontoxic, total FUra-RNA levels.

Therefore, two apparently interrelated factors contribute to the subcellular distribution and accumulation of FUra in RNA: precursor-dependent channeling, and altered rRNA metabolism. FUrd was preferentially incorporated into pre-rRNA and

was found to inhibit rRNA processing at low concentrations, which limited its accumulation in the 28 S and 18 S mature rRNA, the predominant RNA species in cells. However, 5′-dFUrd appears to be preferentially channeled into nucleoplasmic RNA and, although incorporation did occur, also was less able to inhibit rRNA production. Thus, with time, a substantial amount of FUra incorporation from 5′-dFUrd accumulated the mature 28 S and 18 S rRNA. Other studies have suggested an explanation for why these two drugs may affect rRNA processing differently even though both result in FUra incorporation into the 45 S precursor rRNA. The rapid conversion of FUrd to FUTP results in a rapid rate incorporation of this analog into RNA, allowing for a high density of FUra substitution for uracil residues in the newly synthesized RNA transcripts. This high density labeling may in turn induce physiochemical alterations which result in the prevention of processing of RNA to the mature species. Therefore, the net accumulation of FUra in the mature 18 S and 28 S rRNA would be self-limited by this processing block. However, 5′-dFUrd is converted to FUTP at a much slower rate. This, combined with an apparent preferential channeling into other species of nuclear RNA, produces a much lower density of FUra substitution per molecule of newly made rRNA transcripts, which may be insufficient to produce the alterations necessary to block rRNA processing. With time, these less densely labeled RNA molecules would be processed normally and accumulate in the cell as mature rRNA, thereby contributing to the eventual high levels of FUra-containing RNA generated by nonlethal doses of 5′-dFUrd.

The question of which RNA-related lesion is responsible for fluoropyrimidine cytotoxicity was not specifically addressed in these experiments, although this topic is currently under much study. However, in this regard it is of interest that high levels of FUra incorporation into nucleoplasmic RNA (8.4 and 19.1 pmol of FUra/μg of RNA) were observed following the nontoxic 5′-dFUrd treatments (50 and 100 μM 5′-dFUrd, respectively). These levels were much greater than those produced by the toxic FUrd concentrations. Any RNA alteration induced by FUra accumulation in this subcellular fraction following treatment with 5′dFUrd was not lethal to S-180 cells under these conditions. Because nucleoplasmic RNA is reported to contain predominantly pre-mRNA, the current results with 5′-dFUrd would suggest that FUra incorporation into this RNA does not affect cell viability. This is in apparent contrast to the recent reports and speculation by Dolnick and Pink (4, 18) that FUra-altered mRNA mediates dThd-nonreversible cytotoxicity. The latent changes in this species of RNA observed by these investigators would appear to be secondary to other macromolecular changes.

It is apparent that monitoring more specific interactions of FUra with RNA is required for evaluating possible cytotoxic drug actions on RNA metabolism. Measuring the incorporation of FUra into a distinct species or class of RNA may be more closely related to drug toxicity than to the total cell levels of FUra in RNA. Supporting this concept is the observation made by Herrick and Kufe (9) of a highly significant association between FUra incorporation into nuclear pre-rRNA and cell lethality. This may also be important in combination chemotherapy studies which use biochemical modulators to increase FUra activity. Drug treatments which only minimally affect the level of FUra in total cell RNA may actually enhance FUra-induced aberrations in specific RNA species. For example, Major *et al.* (19) examined FUra combinations with methotrexate, *N*-(phosphonacetyl)-L-aspartate and 6-methylmercaptopurine riboside and found that the enhanced FUra cytotoxicity observed after antimetabolite pretreatment weakly correlated with the absolute level of FUra in total cell RNA. However, when only newly synthesized RNA was specifically examined, a highly significant association between incorporation and cytotoxicity was observed.

In summary, our studies have demonstrated that different precursors of FUTP do not necessarily produce similar types of FUra incorporation into RNA. Thus, the absolute level of incorporation of FUra in total cell RNA is a poor indicator of the overall effects of the fluoropyrimidines. Monitoring more specific interactions of FUra with distinct types of RNA is necessary for evaluating the possible cytotoxic actions of this class of drugs.

References

1. Chabner. B. *Pharmacologic Principles of Cancer Treatment.* W. B. Saunders Co., Philadelphia, 183–212 (1982).
2. Kufe, D., and P. Major. 5-Fluorouracil incorporation into human breast carcinoma RNA correlates with cytotoxicity. *J. Biol. Chem.* **256:**9802–9805 (1981).
3. Glazer, R. I., and L. S. Lloyd. Association of cell lethality with incorporation of 5-fluorouracil and 5-fluorouridine into nuclear RNA in human colon carcinoma cells in culture. *Mol. Pharmacol.* **21:**468–473 (1982).
4. Dolnick, B., and J. Pink. 5-Fluorouracil modulation of dihydrofolate reductase RNA levels in methotrexate-resistant KB cells. *J. Biol. Chem.* **258:**13299–13306 (1983).
5. Spigelman, S., R. Nayak, R. Sawyer, R. Stolfi, and D. Martin. Potentiation of the anti-tumor activity of 5FU by thymidine and its correlation with the formation of (5FU)RNA. *Cancer (Phila.)* **45:**1129–1134 (1980).
6. Armstrong, R. D., K. M. Connolly, A. M. Kaplan, and E. Cadman. Mechanism of cytotoxic activity of 5′-deoxy-5-fluorouridine. *Cancer Chemother. Pharmacol.* **11:**102–105 (1983).
7. Cadman, E., R. Heimer, and L. Davis. Enhanced 5-fluorouracil nucleotide formation after methotrexate administration: explanation for drug synergism. *Science (Wash. D. C.)* **205:**1135–1137 (1979).
8. Wilkinson, D., and H. Pitot. Inhibition of ribosomal ribonucleic acid maturation in Novikoff hepatoma cells by 5-fluorouracil and 5-fluorouridine. *J. Biol. Chem.* **248:**63–68 (1973).
9. Herrick, D., and D. Kufe. Lethality associated with incorporation of 5-fluorouracil into preribosomal RNA. *Mol. Pharmacol.* **21:**468–473 (1982).
10. Houghton, J., and P. Houghton. On the mechanism of cytotoxicity of fluorinated pyrimidines in four human colon adenocarcinoma xenografts maintained in immune-deprived mice. *Cancer (Phila.)* **45:**1159–1167 (1980).
11. Takimoto, C., E. Cadman, and R. Armstrong. Differential incorporation of fluorouracil into selected RNA. *Clin. Res.* **32:**423A (1984).
12. Cadman, E., R. Heimer, and C. Benz. The influence of methotrexate pretreatment of 5-fluorouracil metabolism in L1210 cells. *J. Biol. Chem.* **256:**1695–1704 (1981).
13. Penman, S., I. Smith, E. Holtzman, and H. Greenberg. RNA metabolism in the HeLa cell nucleus and nucleolus. *Natl. Cancer Inst. Monogr.* **23:**489–512 (1966).
14. Armstrong, R., and R. Diasio. Metabolism and biological activity of 5′-deoxy-5′-fluorouridine, a novel fluoropyrimidine. *Cancer Res.* **40:**3333–3338 (1980).
15. Genchev, D., M. Kermekchiev, and A. Hadjiolov. Free pyrimidine nucleotide pool of Ehrlich ascites tumor cells. *Biochem. J.* **188:**85–90 (1980).
16. Shani, J., and P. Danenberg. Evidence that intracellular synthesis of 5-fluorouridine-5′-phosphate from 5-fluorouracil and 5-fluorouridine is compartmentalized. *Biochem. Biophys. Res. Commun.* **122:**439–445 (1984).
17. Armstrong, R. D., and E. Cadman. A role for the rate of 5-fluorouracil (FUra) incorporation into RNA in mediating fluoropyrimidine cytotoxicity. *J. Cell. Biochem.* **6:**373 (1982).
18. Dolnick B., and J. Pink. Effects of 5-fluorouracil on dihydrofolate reductase and dihydrofolate reductase mRNA from methotrexate-resistant KB cells. *J. Biol. Chem.* **260:**3006–3014 (1985).
19. Major, P., E. Egan, L. Sargent, and D. Kufe. Modulation of 5-FU metabolism in human MCF-7 breast carcinoma cells. *Cancer Chemother. Pharmacol.* **8:**87–91 (1982).

Send reprint requests to: Dr. R. Douglas Armstrong, Cancer Research Institute, M-1282, University of California, San Francisco, CA 94143.