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The Effect of 5-Fluorouracil on the Synthesis and Methylation of Low Molecular Weight Nuclear RNA in L1210 Cells

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SUMMARY

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The effect of 5-fluorouracil on the synthesis, methylation and its incorporation into low molecular weight nuclear RNA was studied in L1210 cells *in vitro*. 5-Fluorouracil was incorporated into high molecular weight, 5 S and 4 S nuclear RNA at a linear rate for 2 hr following an initial lag period. Drug concentrations of 1×10^{-5} to 1×10^{-4} M did not have a marked effect on the incorporation of [^{3}H]adenosine into 5 S and 4 S nuclear RNA; however, the synthesis of high molecular weight nuclear RNA was inhibited 10–60% depending on the length of time of labeling and the drug concentration. 5-Fluorouracil impaired the methylation of 4 S nuclear RNA in a dose-dependent manner and affected high molecular weight nuclear RNA to a lesser extent. 5-Fluorouracil at 1×10^{-4} M inhibited the methylation of eight methylated nucleosides in 4 S nuclear RNA, but at 5×10^{-5} M preferentially affected 7-methylguanosine, N^2,N^2-dimethylguanosine and 5-methyluridine. These results indicate that the methylation of 4 S nuclear RNA is impaired by 5-fluorouracil at concentrations of drug which do not significantly impair its transcription.

INTRODUCTION

The antitumor effect of 5-fluorouracil has been traditionally ascribed to its ability to inhibit DNA synthesis via blockade of thymidylate synthetase by its metabolite, FdUMP[1](1–3). This biochemical lesion, viz., inhibition of DNA synthesis, has not been well documented since radiolabeled precursors of DNA which do not flux through the thymidylate synthetase pathway are incorporated into DNA at a normal or accelerated rate (1, 2, 4). These results suggest that at least in some tumors, the cytotoxicity of 5-FU may be relegated to the perturbation of other metabolic processes, such as RNA synthesis.

The effect of 5-fluorouracil on the synthesis and processing of RNA in tumor cells has been actively investigated for several years. The incorporation of this antimetabolite into RNA has been shown to correlate with the chemotherapeutic response of the tumor (5–7). Other investigations expounding on this mechanism of action have shown that the degree of sensitivity and resistance of tumors to 5-FU was related to its incorporation into RNA (8–10).

At the molecular level, 5-FU has been shown to inhibit the maturation of precursor rRNA in direct proportion to the amount of drug sensitivity of Novikoff hepatoma cells (11). Interference with the processing of rRNA was recently shown to be augmented by high doses of thymidine (12), a treatment which enhances the incorporation of 5-FU into rRNA and mRNA (12), as well as its effectiveness as an antitumor agent (13).

The action of 5-FU is not confined to rRNA. Poly(A)RNA from nuclei or polyribosomes contained the highest specific radioactivity of labeled 5-FU of all species of RNA examined (14, 15). Hepatic poly(A)RNA from partially hepatectomized rats treated with 5-FU also showed an enhanced translational activity *in vitro* (15).

Thus, 5-FU has the potential for inducing modifications in RNA structure for at least two species of RNA. The mechanism for these changes may be related to base-pair transformations occurring during transcription (16), despite the fact that transcription per se is not inhibited at low concentrations of drug (14, 16, 17).

In contrast to the aforementioned effects of 5-FU on rRNA and mRNA, virtually little is known about the effect of this drug on low molecular species of RNA from

[1] The abbreviations used are: 5-FU, 5-fluorouracil; 5-FdUMP, 5-fluoro-2′-deoxyuridylic acid; nRNA, nuclear RNA; poly(A), polyadenylic acid; SDS, sodium dodecyl sulfate; m^{1}A, 1-methyladenosine; m^{3}C, 3-methylcytosine; m^{5}C, 5-methylcytosine; m^{1}G, 1-methylguanosine; m^{2}G, N^2-methylguanosine; m$_2^2$G, N^2,N^2-dimethylguanosine, m^{7}G, 7-methylguanosine; m^{5}U, 5-methyluridine; 4 S RNA, tRNA.

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mammalian cells. Tseng *et al.* (18) recently reported that 5-FU inhibited 5-methyluracil methyltransferase from the liver and tumor of mice, and that this effect directly accounted for the hypomethylation of 5-methyluracil contained in tRNA. 5-FU-modified tRNA from *Escherichia coli* exhibited low rates of aminoacylation for some amino acids, as well as low levels of polypeptide synthesis (19). Thus far, 5-FU has not been shown to have any inhibitory effects on the synthesis of low molecular weight RNA (12, 20).

The present study was designed to examine the action of 5-FU on low molecular weight nRNA. The ability of 5-FU to affect the synthesis of 5 S and 4 S RNA, as well as the base methylation of 4 S RNA, forms the basis for this report.

MATERIALS AND METHODS

Materials. 5-FU was obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. 5-[6-^{3}H]FU (2 Ci/mmole) was obtained from Amersham/Searle Corporation, Arlington Heights, Illinois, and L-[*methyl*-^{3}H]methionine (80 Ci/mmole) and [2,8-^{3}H]adenosine (31 Ci/mmole) were purchased from New England Nuclear Corporation, Boston, Massachusetts. m^1A, m^3C, m^5C, m^1G, $m_2{}^2G$, m^7G and m^5U were obtained from P-L Biochemicals, Milwaukee, Wisconsin.

Animals. L1210 cells were inoculated i.p. into BALB/c × DNA/2 F_1 mice at an inoculum of 10^5 cells/0.1 ml Hanks' balanced salt solution. Cells were harvested 6 days after inoculation and were further diluted with incubation medium to 2×10^7 cells/ml.

Incubations. Incubations of L1210 cells were carried out at 37° in a shaking water bath at 100 rpm and consisted of: (1) 25 ml of RPMI 1630 medium, 5×10^7 cells and either 50 μCi [^{3}H]adenosine (31 Ci/mmole) or 100 μCi 5-[^{3}H]FU (400 mCi/mmole); or (2) 25 ml of L-methionine-free RPMI 1630, 5×10^7 cells and 250 μCi [*methyl*-^{3}H]methionine (80 Ci/mmole). Cells were incubated with unlabeled 5-FU for 30 min before addition of isotopes and the period of labeling was varied as indicated in the text.

RNA extraction. After incubation, cells were centrifuged at 400*g* for 20 min and washed once with incubation medium. Nuclei were prepared as previously described (14), except that cells were swelled in 10 mM magnesium acetate (pH 5.1). This change in swelling medium was found to markedly arrest the activity of intracellular RNase, and thereby minimize hydrolysis of nRNA before extraction. nRNA was extracted from nuclei with 3 ml of 0.1% SDS:0.14 M NaCl:0.025 M sodium acetate (pH 5.1) and 3 ml of phenol mixture (phenol:*m*-cresol:water, 7:2:2, v/v, containing 0.1% 8-hydroxyquinoline) by vortexing vigorously for 2 min. The emulsion was clarified by centrifugation at 12,000*g* for 10 min and the upper aqueous phase removed and precipitated with 3 vol of 2% potassium acetate in 95% ethanol at −20° overnight.

Electrophoresis. nRNA was resolved by electrophoresis in cylindrical polyacrylamide gels (0.4 × 7 cm) containing: 8% (w/v) acrylamide, 0.32% (w/v) diallytartardiamide, 6 M urea, 0.1% (w/v) SDS, 0.2% (w/v) ammonium persulfate, 0.04% (v/v) *N,N,N'N'*- tetramethylenediamine, 0.4 M Tris–acetic acid (pH 7.2), 0.02 M sodium acetate and 0.002 M EDTA. RNA samples containing one A_{260} unit were mixed with sample buffer to give a final concentration of 0.04 M Tris–acetic acid (pH 7.2), 0.02 M sodium acetate, 0.002 M EDTA, 0.02% bromphenol blue and 20% (w/v) sucrose (RNase-free). Gels were electrophoresed at 4 M per gel at 4°. Gels were sectioned into 2-mm slices, dissolved in 2% (w/v) periodic acid at 37° for 15 min, mixed with 10 ml of Aquasol (New England Nuclear) and counted in a Searle Mark III liquid scintillation spectrometer. Gels were stained with 2% methylene blue dissolved in 15% acetic acid for 1 hr and destained by diffusion in 15% acetic acid.

Thin-layer chromatography. The 4 S RNA was isolated by polyacrylamide gel electrophoresis, sliced from the appropraprite section of the gel and sectioned into 2-mm slices. RNA was extracted from four gel slices with 1 ml of RNA extraction buffer (0.1% SDS:0.014 M NaCl: 0.025 M sodium acetate, pH 5.1) and continuous vortexing at room temperature for 1 hr. The gel was removed by centrifugation at 16,000 *g* for 2 min in an Eppendorf centrifuge, and the RNA was precipitated at −20° for 2 hr. Enzymatic digestion was carried out for 18 hr at 37° in 20 μl of 0.05 M Tris–Hcl (pH 8.0):5 mM $MgCl_2$ containing:6 μg RNase A, 5 μg calf intestine alkaline phosphatase (1000 units/mg) and 10 μg snake venom phosphodiesterase. Samples were freeze-dried and reconstituted with 20 μl of a standard mixture containing the eight methylated nucleosides at a concentration of 0.4 mg/ml. An aliquot

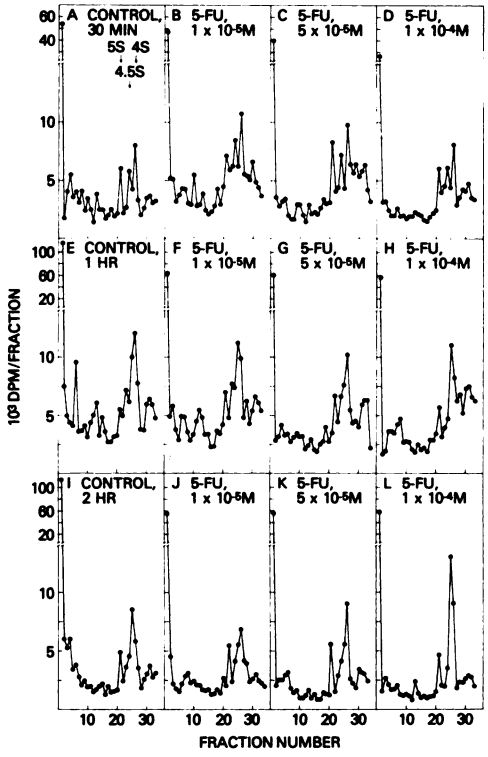

FIG. 1. *Incorporation of [^{3}H]adenosine into nRNA in the presence of 5-FU*

L1210 cells were incubated for 30 min with 5-FU at the indicated concentrations and further incubated with 50 μCi of [^{3}H]adenosine for 30 min (Panels A–D), 1 hr (Panels E–H) and 2 hr (Panels I–L). RNA was extracted from isolated nuclei and electrophoresed in 8% polyacrylamide gels as described under Materials and Methods.

of 5 μl was spotted on 0.25-mm silica gel plates containing fluorescent indicator (EM Laboratories, Elmsford, N.Y.) and the eight methylated nucleosides were separated by two-dimensional thin-layer chromatography with acetonitrile:concentrated NH_4OH (4:1, v/v) for the first dimension and acetonitrile:2 N HCOOH (10:1, v/v) for the second dimension (18).

RESULTS

Initial dose-response studies were carried out with [^{3}H]adenosine as precursor using varying times of pulse labeling (Fig. 1). nRNA subjected to electrophoresis in denaturing 8% polyacrylamide gels was resolved into five major species of low molecular weight nRNA as assessed with methylene blue stain (data not shown). Of the classes of nRNA visualized in the gels, only 4 S, 4.5 S and 5 S nRNA showed significant labeling (Figs. 1A, E, I). The synthesis of 4 S and 5 S nRNA as assessed by the area of radioactivity associated with each peak was not affected by 5-FU at concentrations of 1×10^{-5} to 1×10^{-5} M. In contrast, the labeling of high molecular weight nRNA (at the top of the gel) was diminished by 5–25% at 30 min of labeling (Figs. 1B–D) and by 45% at longer labeling times (Figs. 1F–H, J–L).

The incorporation of 1×10^{-5} M 5-[^{3}H]FU into 5 S and 4 S nRNA was measured at various incubation times (Figs. 2 and 3). After a lag period of 30 min, incorporation of drug into 5 S and 4 S nRNA proceeded at a linear rate. After 1 hr of incubation, 5-[^{3}H]FU was clearly evident in both fractions of RNA with the incorporation of radioactivity approximately threefold higher in 4 S versus 5 S RNA.

Since 5-FU was efficiently incorporated into 5 S and 4 S nRNA at concentrations that did not affect their transcription, the possibility that the antimetabolite interfered with posttranscriptional methylation of 4 S nRNA was investigated. As expected, only 4 S nRNA showed extensive labeling when L1210 cells were incubated for 1 hr with [*methyl*-^{3}H]methionine (Fig. 4). 5-FU at 1×10^{-5}, 5×10^{-5} and 1×10^{-4} M produced approximately 20, 40 and 50% inhibition of labeling of 4 S nRNA, but 0, 15 and 30% inhibition, respectively, of high molecular weight nRNA.

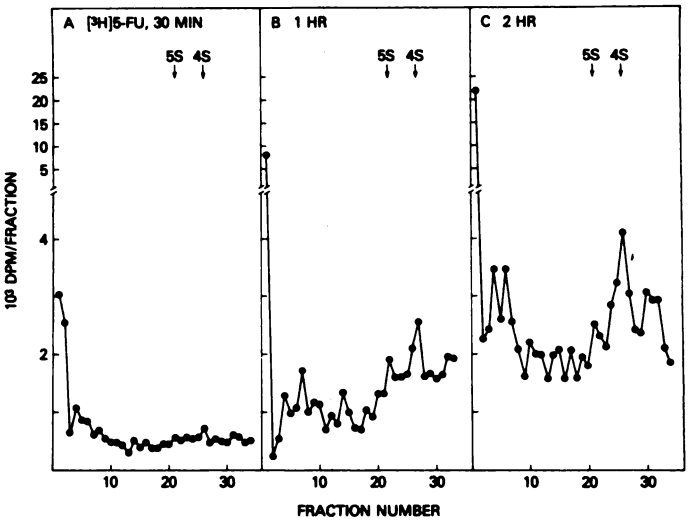

FIG. 2. *Polyacrylamide gel electrophoresis of nRNA labeled with [^{3}H]5-FU*

L1210 cells were incubated with 1×10^{-5} M [^{3}H]5-FU (400 mCi/mmole) for the indicated periods of time, and nRNA was extracted from isolated nuclei as described under Materials and Methods.

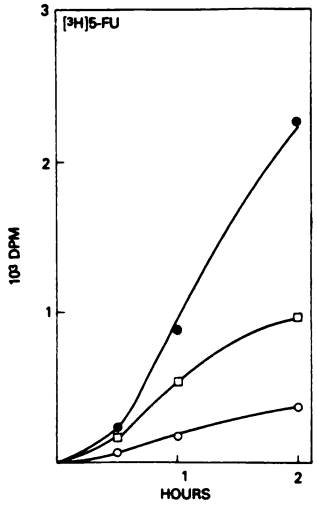

FIG. 3. *Time course of incorporation of [^{3}H]5-FU into snRNA*

L1210 cells were incubated with [^{3}H]5-FU as described in Fig. 2, and high molecular weight nRNA (●), 5 S RNA (○), and 4 S nRNA (□) were extracted from isolated nuclei and separated by electrophoresis as described under Materials and Methods.

To further assess the specificity of the effect of 5-FU on the methylation of 4 S nRNA, this RNA fraction was purified by electrophoresis, extracted from the gel, enzymatically hydrolyzed and methylated nucleosides were separated by two-dimensional thin-layer chromatography (Table 1). While no statistically significant reduction in the base methylation of the eight methylated nucleosides of 4 S nRNA occurred at 1×10^{-5} M 5-FU, specific inhibition of m^7G, m_2^2G, m^5U and to a lesser extent, m^2G resulted at 5×10^{-5} M of inhibitor. At the highest concentration of 5-FU, significant reduction in methylation of all methylated nucleosides occurred with m^5U showing the largest degree of inhibition.

DISCUSSION

The effect of 5-FU on RNA synthesis and processing may be crucial for its cytotoxic action on sensitive tumors. Substantial evidence has accumulated relating the incorporation of 5-FU into RNA and the degree of sensitivity or resistance of the cell (4–8, 13). Previous studies were directed toward assessing the effect of this antimetabolite on the processing of nucleolar RNA where it was shown that the conversion of precursor rRNA to 28 S and 18 S rRNA was more sensitive than transcription per se (11). The transcription of various species of nRNA in L1210 cells including rRNA and poly(A)RNA were relatively insensitive to the effects of low concentrations (less than 1×10^{-4} M) of 5-FU (14). The present report also extends this phenomenon to 4 S and 5 S nRNA where their transcription remained unimpaired at 1×10^{-4} M 5-FU during prolonged periods of labeling. Thus, it appears that either the incorporation of 5-FU into RNA to alter its function or posttranscriptional modification of RNA

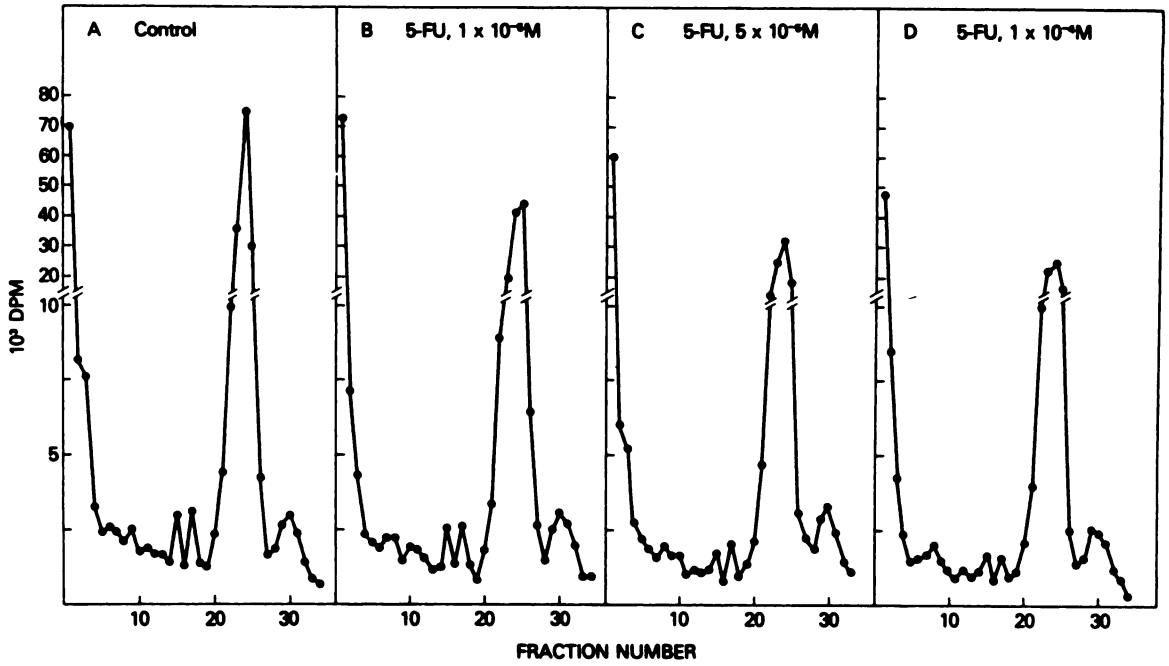

FIG. 4. *Methylation of 4 S RNA in the presence of 5-FU*

L1210 cells were incubated for 30 min with 5-FU at the indicated concentrations and further incubated with 250 μCi of L-[*methyl*-^{3}H]methionine (80 Ci/mmole) for 1 hr. RNA was extracted from isolated nuclei and electrophoresed in 8% polyacrylamide gels as described under Materials and Methods.

or both are responsible for the aberrant effects of this antimetabolite on nRNA. With respect to altered 4 S RNA function, 5-FU-modified bacterial tRNA exhibited reduced rates of aminoacylation for specific amino acids (19). The reason for this phenomenon was not elucidated, nor has this feature of 5-FU action been examined for mammalian tRNA. However, our data indicate that L1210 cells are capable of incorporating 5-FU into 4 S and 5 S nRNA over prolonged periods of time.

Other studies have stressed the posttranscriptional process of methylation (18). Experiments with a mouse mammary tumor have documented that 5-FU specifically interferes with the methylation of m^5U *in vivo* (18). Our results agree, in part, with these studies in that m^5U appears to be most sensitive to the inhibitory effects of 5-FU on the methylation of 4 S nRNA. However, its effects were not limited to m^5U, and in fact, the labeling of all methylated bases in 4 S nRNA was reduced at the highest concentration of 5-FU tested. Thus, it is possible that a deficit in base methylation can alter the tertiary structure of 4 S nRNA, and hence its ability to function normally in translation.

With regard to other posttranscriptional modifications of nRNA, 5-FU impairs polyadenylylation of nRNA to a slightly greater degree than the methylation of 4 S nRNA, while the methylation of ribosomal nRNA shows a lesser degree of sensitivity to 5-FU (14). Although it appears that one of the major effects of 5-FU may be on

TABLE 1

The effect of 5-FU on the methylation of 4 S RNA

L1210 cells were incubated with 5-FU for 30 min and further incubated with L-[*methyl*-^{3}H]methionine for 1 hr. RNA was extracted from isolated nuclei and electrophoresed in 8% polyacrylamide gels. 4 S RNA was extracted from the gels, enzymatically digested, and methylated nucleosides were separated by two-dimensional thin-layer chromatography as described under Materials and Methods. Each value is the mean ± SE from four determinations.

Treatment	Methylated nucleoside							
	m^7G	m^5C	m^1A	m^1G	m_2^2G	m^5U	m^2G	m^3C
	(dpm/5 × 10^7 cells)							
Control	1710 ± 170	600 ± 50	320 ± 40	1180 ± 90	5380 ± 670	1160 ± 110	2480 ± 80	210 ± 20
	(% of control)							
5-FU								
1×10^{-5} M	72 ± 20	92 ± 6	90 ± 10	93 ± 10	68 ± 8	77 ± 13	99 ± 3	87 ± 6
5×10^{-5} M	52 ± 11[a]	81 ± 7	70 ± 11	81 ± 6	53 ± 6[a]	40 ± 3[c]	70 ± 9[a]	84 ± 9
1×10^{-4} M	41 ± 9[a]	72 ± 3[a]	53 ± 10[a]	60 ± 5[a]	35 ± 4[b]	26 ± 2[c]	47 ± 6[c]	50 ± 4[b]

[a] Statistically significant difference ($p < 0.05$) *vs* control.
[b] Statistically significant difference ($p < 0.01$) *vs* control.
[c] Statistically significant difference ($p < 0.001$) *vs* control.

the processing of nRNA rather than on its synthesis, it is still not clear what consequences this will have on the ability of the target cell to function normally. One result directly attributable to 5-FU-modified RNA is the increased translational activity *in vitro* of poly(A)RNA isolated from partially hepatectomized rats treated with this drug during the early and late G_1 phase of cell growth (15). Perhaps even more significant to consider is the totality of the effect of 5-FU-modified rRNA, 4 S RNA and mRNA on the translational process. It will be interesting to see if one or more of these species of RNA contribute more significantly to the tumoricidal effects of 5-FU.

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