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Cytotoxicity and the Inhibition of Ribosomal RNA Processing in Human Colon Carcinoma Cells

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SUMMARY

The effects of six nucleoside and base analogs, 5-fluorouracil, 5-azacytidine, sangivamycin, toyocamycin, 8-azaguanine, and tubercidin, on ribosomal RNA processing and cell viability were examined in the colon carcinoma cell line HT-29. Exposure of HT-29 cells to various concentrations of each of these compounds for 24 hr produced two distinct types of results. Toyocamycin, 5-fluorouracil, and tubercidin caused an exponential type of cell lethality resulting in 3–4 log reduction of cell viability, while sangivamycin, 8-azaguanine, and 5-azacytidine produced a gradual and self-limiting type of cell lethality resulting in no greater than a 1 log reduction of cell viability. Likewise, the effects of these drugs on rRNA processing resulted in their classification into two groups: toyocamycin, 5-fluorouracil, and tubercidin caused an abnormal accumulation of the 45 S precursor to rRNA, while sangivamycin, 8-azaguanine, and 5-azacytidine did not cause an accumulation of 45 S RNA. Sangivamycin, 8-azaguanine, and 5-azacytidine all produced an inhibitory effect on protein synthesis, while tubercidin inhibited protein synthesis at a concentration similar to that which caused the accumulation of 45 S RNA, and toyocamycin and 5-fluorouracil had no effect on protein synthesis at concentrations at which 45 S RNA accumulated. These results show that cells are much less capable of resuming normal proliferative activity after exposure to nucleoside or base analogs which cause the accumulation of 45 S rRNA precursor, than to those which act by other mechanisms.

INTRODUCTION

The initial transcription of mammalian ribosomal RNA is a 45 S precursor that contains in order from the 5′ end, an external transcribed spacer, 18 S rRNA, an internal transcribed spacer, 5.8 S rRNA, a second internal transcribed spacer, and 28 S rRNA. A series of endonucleolytic cleavages at specific sites produces characteristic intermediates and finally mature rRNAs (1). The most abundant intermediates are a 41 S fragment which does not contain the external transcribed spacer and a 32 S fragment containing 5.8 S RNA, the second internal spacer, and 28 S rRNA (2).

Several pyrimidine and purine base and nucleoside analogs including 5-fluorouracil, 5-fluorocytidine, 5-fluoroorotic acid, 5-azacytidine, sangivamycin, 8-azaguanine, 6-thioguanosine, toyocamycin, tubercidin, cordycepin, and xylosyladenine have been shown to inhibit the formation of mature 28 S and 18 S ribosomal RNA (3–17). Cordycepin and xylosyladenine are examples of compounds that appear to inhibit RNA synthesis by either competing with ATP for RNA polymerase or causing premature RNA chain termination (9–12). However, several of the remaining compounds are examples of agents that allow the synthesis of the complete 45 S precursor rRNA but that then inhibit the 45 S precursor rRNA from being properly transformed into mature rRNA (3–8, 14).

The significance of the contribution of the inhibition of rRNA processing towards the mechanism by which these compounds kill cells has never been determined. In this report, we compare the cytotoxicity resulting from several compounds that incorporate into RNA to their ability to inhibit rRNA processing.

MATERIALS AND METHODS

Materials. [5-^{3}H]Urd (26.2 Ci/mmol) and [U-^{4}C]Urd (506 mCi/mmol) were purchased from New England Nuclear Corporation (Boston, MA). 5-Fluorouracil, 5-azacytidine, sangivamycin, toyocamycin, 8-azaguanine, and tubercidin were provided by the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD).

Cell Culture. The growth properties of human colon carcinoma cell line HT-29 have been described previously (18). Cells were grown in monolayers at 37° and 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, gentamycin, 50 mg/ml, and 40 mM Hepes,[1] pH 7.4. Cell inocula consisted of 10^5 cells/10 ml in a T-25 flask or 10^6 cells/100 ml in a T-150 flask and were allowed 3 days

[1] The abbreviations used are: Hepes; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium lauryl sulfate.

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to enter logarithmic growth before drugs were added. Cells for RNA extraction were prelabeled for 48 hr with 0.025 μCi/ml of [^{4}C]Urd (506 mCi/mmol) after which the medium was replaced and the cells were then labeled for 24 hr with 0.2 μCi/ml of [^{3}H]Urd (500 mCi/mmol) concurrent with drug treatment.

Cell viability determination. The soft agar clonogenic assay was performed as previously described (19) except that the cloning medium contained 20% fetal calf serum and 40 mM Hepes, pH 7.4. Cell viability is expressed as the number of surviving colonies from drug-treated cells/the number of colonies from control cells × 100. Cloning efficiency ranged from 30 to 40%.

Protein synthesis. The rate of protein synthesis was determined by the incorporation of [^{3}H]leucine. Cells were labeled with 1.0 μCi/ml of [^{3}H]leucine (initial specific activity, 146 Ci/mmol; specific activity after dilution by leucine in the medium, 2.6 mCi/mmol) during the last hour of a 6-hr drug treatment, trypsinized, washed with medium, precipitated with cold 10% trichloroacetic acid/2% pyrophosphate, and collected on glass filter discs (GF/B, Whatman Laboratory Products, Clifton, NJ). Radioactivity was determined by liquid scintillation counting.

Extraction of whole cell RNA. Cell monolayers were washed once, scraped into ice-cold phosphate-buffered saline (6.3 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, 0.154 M NaCl, pH 7.4), and collected by centrifugation. The cells were resuspended in 1.8 ml of extraction buffer (20 mM sodium acetate, pH 5.0, 0.14 M NaCl, polyvinyl sulfate, 10 μg/ml) and made 0.2% in SDS (200 μl of 2% SDS) while vortexing at room temperature. After 1 min, 2 ml of phenol (water-saturated containing 0.1% 8-hydroxyquinoline) was added, vortexed for 2 min, and centrifuged at 10,000 × *g* for 5 min to separate the phases. The aqueous phase and protein interface were reextracted with fresh phenol; finally, the aqueous phase was reextracted with fresh phenol for a third time. Three volumes of 95% ethanol/2% potassium acetate were added and the RNA precipitated at −20° overnight. The RNA was collected by centrifugation at 10,000 × *g* for 10 min and washed once with 5 ml of 95% ethanol. Samples were dissolved in 100 μl of gel electrophoresis loading buffer and 0.2 A_{260} unit was loaded per gel.

Gel electrophoresis. RNA was separated on 11-cm cylindrical gels containing 1.9 to 2.1% polyacrylamide/bisacrylamide (19:1) and 0.6% agarose in buffer (40 mM Tris, 20 mM sodium acetate, 3 mM EDTA, 10% glycerol, pH 7.6) (20). Running buffer consisted of the above buffer plus 0.3% SDS. Loading buffer consisted of running buffer containing 20% glycerol. Gels were run for 6 mA/gel for 3 hr. Gel slices were dissolved in 400 μl of concentrated perchloric acid, neutralized with 200 μl of 8 N NaOH, and measured for radioactivity by liquid scintillation counting. (Note: gels containing greater than 2.1% polyacrylamide will not dissolve in perchloric acid.)

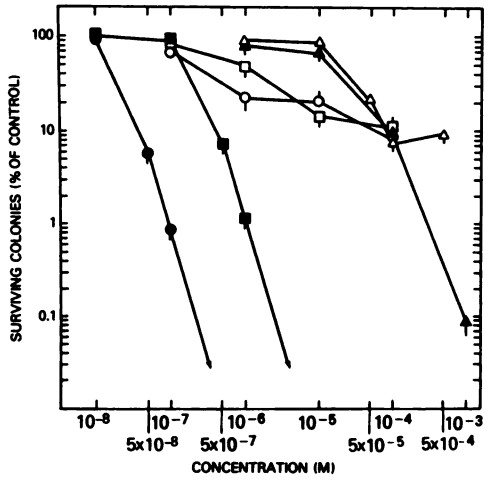

FIG. 1. *Viability of HT-29 cells after exposure to base and nucleoside analogs for 24 hr*

Cells were exposed for 24 hr to toyocamycin (●), tubercidin (■), 5-azacytidine (□), sangivamycin (○), 5-fluorouracil (▲), or 8-azaguanine (△), and viability was determined by soft agar colony formation as described in Materials and Methods. Each value is the mean ± standard deviation of four determinations.

RESULTS

Cell viability. The viability of HT-29 cells exposed for 24 hr to various concentrations of each of the six compounds studied, toyocamycin, tubercidin, sangivamycin, 5-azacytidine, 5-fluorouracil, and 8-azaguanine, was determined by a soft agar clonogenic assay (Fig. 1). The six agents belong to two distinct groups. Toyocamycin, tubercidin, and 5-fluorouracil caused a sharp decrease of viability over a narrow concentration range. Both toyocamycin and tubercidin caused a 4 log reduction of viability, the detection limit of this assay. 5-Fluorouracil caused a reduction of viability of greater than 3 logs at the maximum concentration tested. In contrast, the loss of viability resulting from exposure to sangivamycin, 5-azacytidine, or 8-azaguanine did not surpass 1 log.

Effects on ribosomal RNA processing. Total RNA from cells labeled with [^{3}H]Urd and incubated with increasing concentrations of each of the six compounds was analyzed by polyacrylamide-agarose gel electrophoresis in order to observe their effects on rRNA processing. After 2 hr of labeling with 1 μCi/ml of [^{3}H]Urd, untreated cells

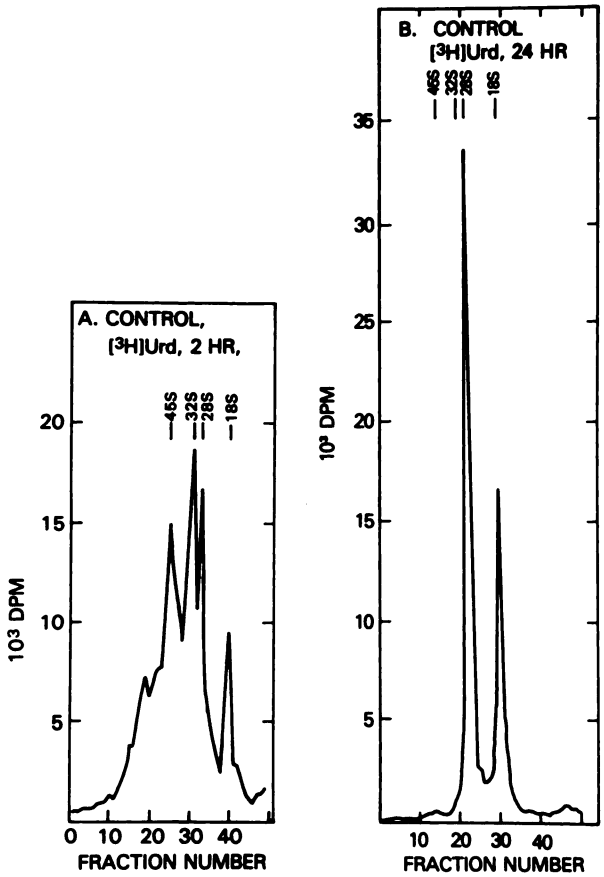

FIG. 2. *Labeling patterns of rRNA in untreated cells*

A, cells were labeled for 2 hr with 1 μCi/ml of [^{3}H]Urd (26.2 Ci/mmol). B, cells were labeled for 24 hr with 0.2 μCi/ml of [^{3}H]Urd (500 mCi/mmol). Total RNA was extracted and analyzed as described in Materials and Methods.

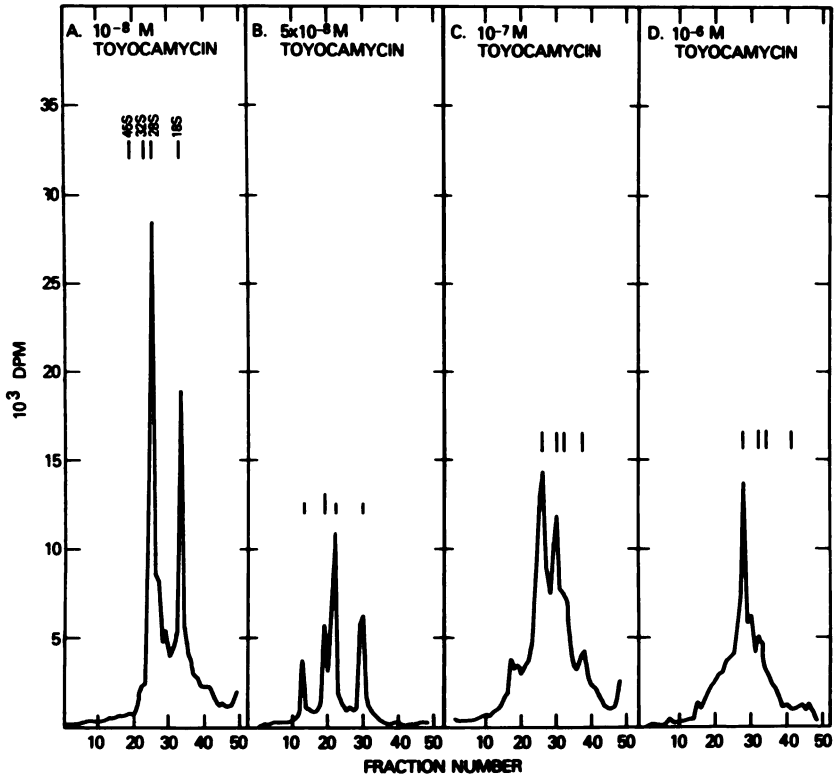

FIG. 3. *Effect of toyocamycin on rRNA processing*
Cells were labeled for 24 hr with 0.2 μCi/ml of [³H]Urd (500 mCi/mmol) concurrent with incubation with toyocamycin. Total RNA was extracted and analyzed as described in Materials and Methods.

contained significant amounts of radioactive rRNA precursors (45 S and 32 S) and mature rRNA (28 S and 18 S) (Fig. 2A). There was also some labeling of high molecular weight material (60 S–90 S) which is probably heterogeneous RNA. However, in cells labeled for 24 hr with 0.2 μCi/ml of [³H]Urd (500 mCi/mmol), the amount of labeled mature RNA continuously accumulated while the amount of labeled precursor RNA and heterogeneous RNA reached a low steady state level and remained there (Fig. 2B). Therefore, significant radioactivity was only observed in mature RNA (28 S and 18 S). The later protocol allowed easy detection of perturbations in rRNA processing and was therefore used in subsequent experiments.

The effect of increasing concentrations of toyocamycin on rRNA is shown in Fig. 3. Toyocamycin at a concentration of 10 nM resulted in a labeling distribution like that observed in untreated cells. Cells incubated with 50 nM toyocamycin accumulated some rRNA precursors, while still forming significant amounts of mature rRNA. Cells treated with 100 nM toyocamycin accumulated greater amounts of 45 S and 32 S precursor and formed no mature rRNA. Finally, at 1 μM toyocamycin, only the 45 S initial transcript was observed. The concentration range within which this inhibition of rRNA processing occurs is identical to the range within which loss of cell viability is observed (Fig. 1).

The effect of increasing concentrations of 5-fluorouracil on rRNA processing is shown in Fig. 4. 5-Fluorouracil at a concentration of 10 μM had no effect on the distribution of rRNA. Cells incubated with 100 μM 5-fluorouracil accumulated both 45 S and 32 S rRNA

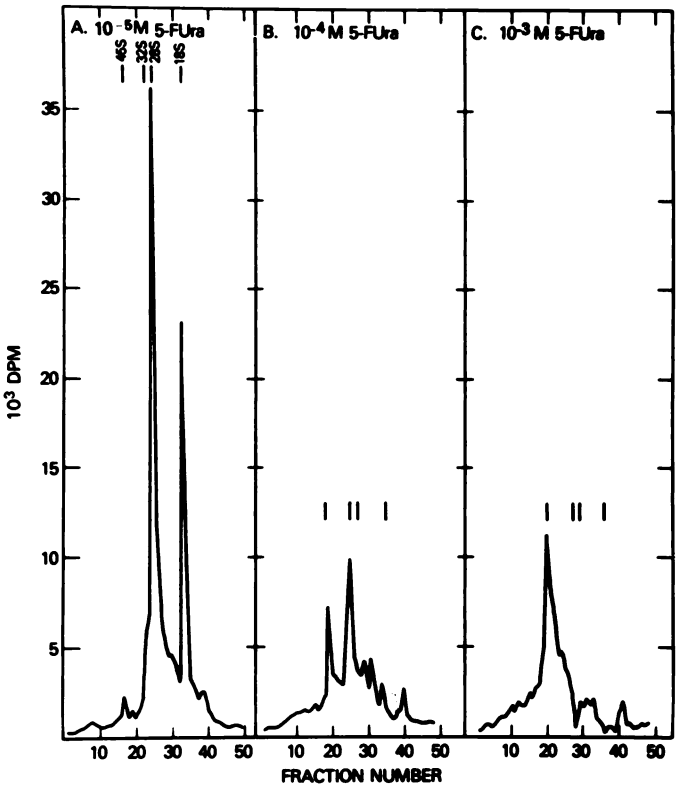

FIG. 4. *Effect of 5-fluorouracil on rRNA processing*
Cells were labeled for 24 hr with 0.2 μCi/ml of [³H]Urd (500 mCi/mmol) concurrent with incubation with 5-fluorouracil (*5-FUra*). Total RNA was extracted and analyzed as described in Materials and Methods.

precursors, while almost no mature rRNA was observed. Cells incubated with 1 mM 5-fluorouracil accumulated only 45 S rRNA. As with toyocamycin, the concentration range over which the inhibition of rRNA processing occurs is identical to that over which loss of cell viability is observed (Fig. 1).

Tubercidin produced an effect similar to that of toyocamycin (Fig. 5). Tubercidin at 100 nM, a concentration which had no effect on cell viability, resulted in a rRNA profile like that of untreated cells. However, 1 μM tubercidin, a cytocidal concentration, caused inhibition of the processing of rRNA and accumulation of 45 S rRNA.

The effect of increasing concentrations of sangivamycin on rRNA processing is shown in Fig. 6. Incubation of cells with either 1 or 10 μM sangivamycin caused only a minimal accumulation of rRNA precursor, while the amount of mature rRNA is similar to that of control cells. Sangivamycin at 100 μM caused a drastic decrease of the incorporation of [^{3}H]Urd into rRNA. The specific radioactivity of UTP was measured and found to be 75% of that in untreated cells (results not shown). Therefore, the reduced labeling of RNA indicates a lack of transcription rather than an inability to form [^{3}H]UTP from [^{3}H]Urd. No inhibition of processing was observed.

8-Azaguanine produced a similar effect, wherein a concentration of 500 μM did not have any effect on rRNA processing (Fig. 7).

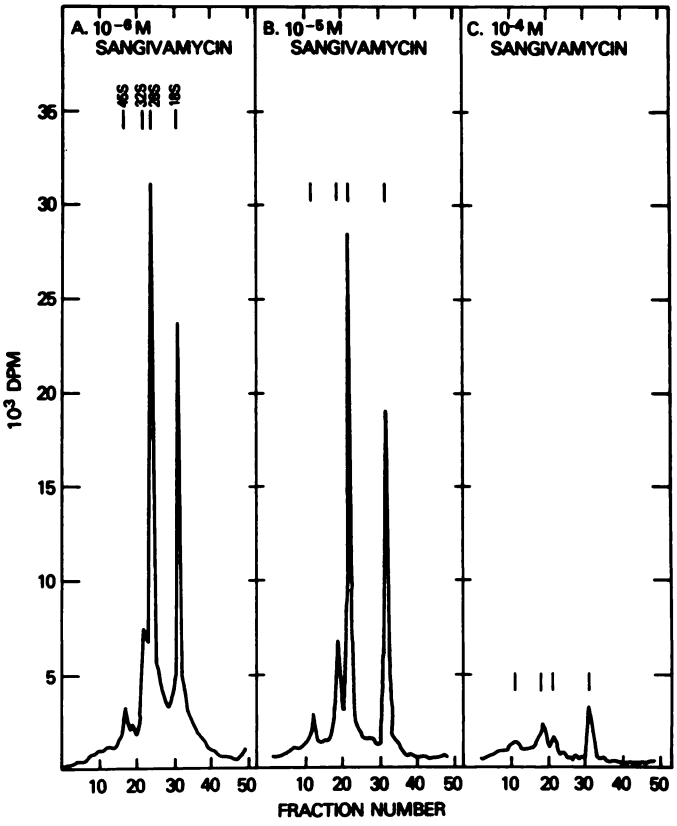

FIG. 6. *Effect of sangivamycin on rRNA processing*
Cells were labeled for 24 hr with 0.2 μCi/ml of [^{3}H]Urd (500 mCi/mmol) concurrent with incubation with sangivamycin. Total RNA was extracted and analyzed as described in Materials and Methods.

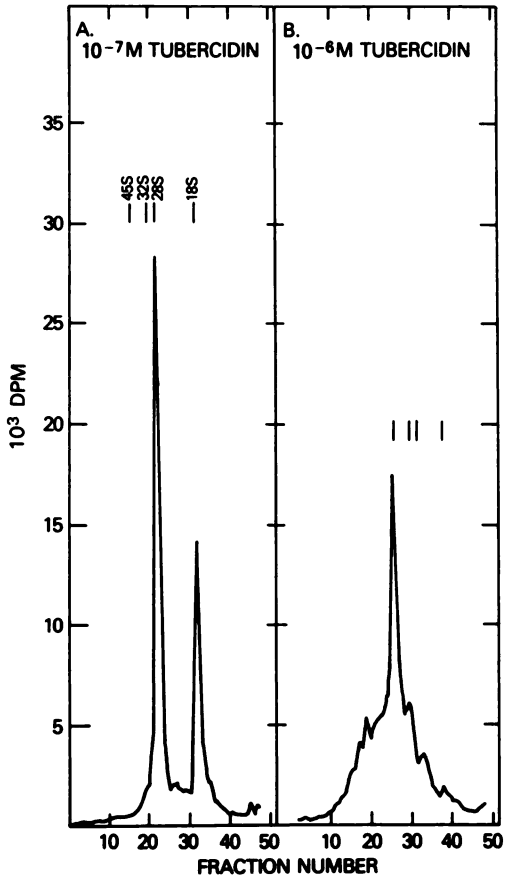

FIG. 5. *Effect of tubercidin on rRNA processing*
Cells were labeled for 24 hr with 0.2 μCi/ml of [^{3}H]Urd (500 mCi/mmol) concurrent with incubation with tubercidin. Total RNA was extracted and analyzed as described in Materials and Methods.

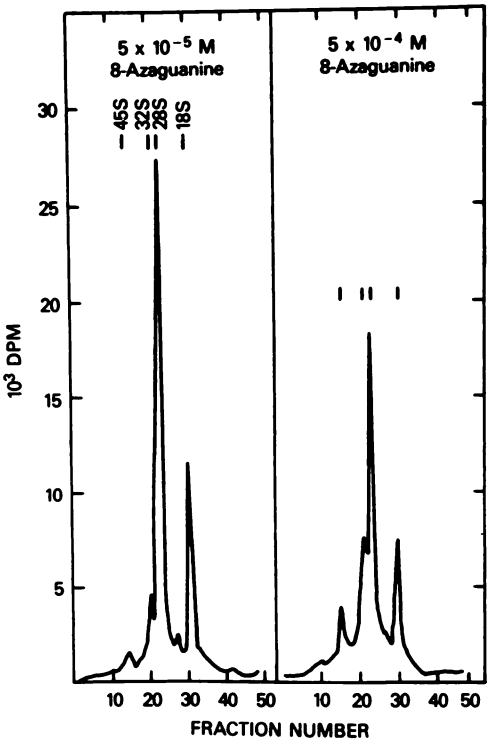

FIG. 7. *Effect of 8-azaguanine on rRNA processing*
Cells were labeled for 24 hr with 0.2 μCi/ml of [^{3}H]Urd (500 mCi/mmol) concurrent with incubation with 8-azaguanine. Total RNA was extracted and analyzed as described in Materials and Methods.

The effect of increasing concentrations of 5-azacytidine on rRNA processing is shown in Fig. 8. 5-Azacytidine at a concentration of 10 μM had no effect on the distribution of rRNA. Cells incubated with 50 μM 5-azacytidine accumulated the 32 S rRNA precursor, but not the 45 S initial transcript. Mature 18 S rRNA was present, but mature 28 S rRNA, which is derived from 32 S rRNA, was not. Cells incubated with 100 μM 5-azacytidine had the same distribution of rRNA, but with low incorporation of precursor. The low level of [^{3}H]Urd incorporated into RNA resulted from a low specific radioactivity of [^{3}H]UTP as a result of the competition between 5-azacytidine and [^{3}H]Urd for phosphorylation (results not shown). Therefore, cells were labeled with [^{3}H]Ado in the presence of 100 μM 5-azacytidine and 1 μM deoxycoformycin, an adenosine deaminase inhibitor. These cells also accumulated [^{3}H]Ado in 32 S rRNA but not 45 S rRNA (results not shown).

Protein synthesis. The effect of each of the six compounds on protein synthesis in HT-29 cells was determined by the incorporation of [^{3}H]leucine (Table 1). Toyocamycin and 5-fluorouracil had minimal effects on protein synthesis (<20% reduction) even at concentrations which caused massive cell kill and inhibition of the processing of the 45 S rRNA precursor. Tubercidin inhibited protein synthesis by 60% at the same concentration range which led to a reduction in cell viability and rRNA processing. Sangivamycin, 8-azaguanine, and 5-azacytidine each inhibited protein synthesis to a significant extent at concentrations which did not cause an accumulation of 45 S on precursor rRNA (Table 1).

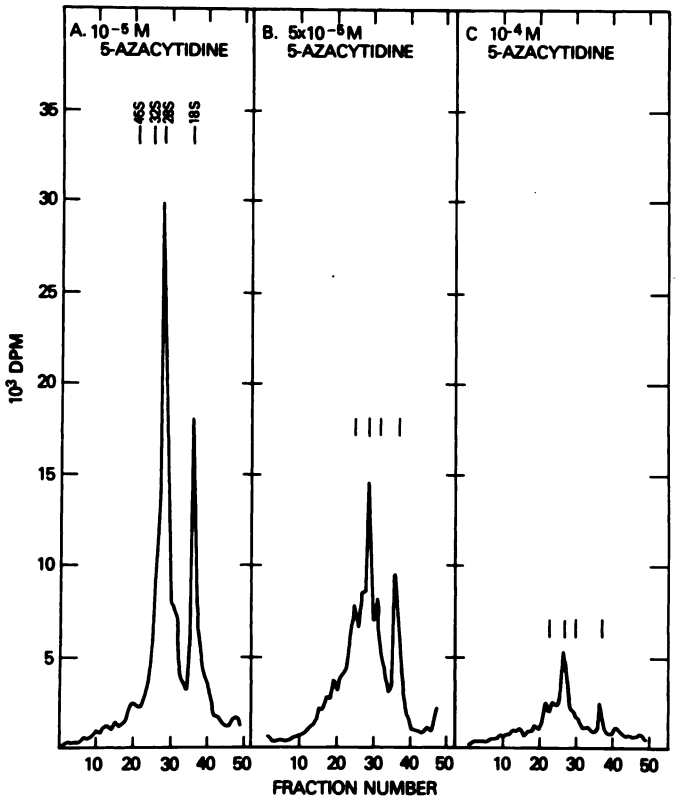

FIG. 8. *Effect of 5-azacytidine on rRNA processing*
Cells were labeled for 24 hr with 0.2 μCi/ml of [^{3}H]Urd (500 mCi/mmol) concurrent with incubation with 5-azacytidine. Total RNA was extracted and analyzed as described in Materials and Methods.

TABLE 1

Effect of a 6-hr exposure to drug on protein synthesis

HT-29 cells were incubated in the presence or absence of drugs for 6 hr, and 1 μCi/ml [^{3}H]leucine was added during the final hour of treatment. Incorporation of [^{3}H]leucine was determined as described under Materials and Methods and is expressed for drug-treated cultures as a percentage of the incorporation obtained in untreated cultures (21,000 dpm). Each value is the mean of duplicate determinations which did not vary by more than 10%.

Drug	Concentration	Incorporation of [^{3}H]leucine
	μM	*% control*
5-Azacytidine	10	12
	50	3
8-Azaguanine	50	36
	500	34
Sangivamycin	0.1	80
	1	41
	10	40
Tubercidin	0.1	85
	1	42
Toyocamycin	0.1	83
	1	79
5-Fluorouracil	100	89
	1000	79

DISCUSSION

The nucleoside and base analogs which incorporate into RNA appear to form two classes. The first group, characterized by toyocamycin, 5-fluorouracil, and tubercidin, produce their primary effect via incorporation into RNA and the inhibition of rRNA processing. The inhibition of rRNA processing by base and nucleoside analogs also has been demonstrated in HeLa, Novikoff hepatoma, L5178Y cells, and regenerating liver (6, 8, 14, 20). Theories on the mechanism by which these compounds inhibit processing of rRNA include: 1) an inability of the 45 S initial transcript to associate properly with required proteins and 2) changes in rRNA conformation induced by analog incorporation such that rRNA-processing enzymes do not function properly (8). Studies involving base and nucleoside analogs have not yet found any difference between the protein content of ribonucleoprotein particles formed in treated versus untreated cells (13, 21). Nondenatured 45 S rRNA from cells treated with either 5-fluorocytidine or 5-azacytidine had a different electrophoretic mobility than that from untreated cells, but that difference disappeared under denaturing conditions (13, 16). This result implies, but does not necessarily prove, that drug substitution resulted in a change in conformation related to the hydrogen bonding involved in base pairing. There are extensive double-stranded regions in precursor rRNA (22, 23), and denaturation of these regions resulting from incorporation of analogs could disrupt rRNA processing. However, such a change in electrophoretic mobility has not been observed with 5-fluorouracil (13).

The second group of nucleoside and base analogs which incorporate into RNA, characterized by sangivamycin, 8-azaguanine, and 5-azacytidine, do not accumulate 45 S rRNA precursor, although 5-azacytidine does inhibit the processing of the 32 S rRNA precursor. Their most prominent effect is a drastic inhibition of protein synthesis caused by a decrease in the formation of complexes

involved in the initiation of protein synthesis (24–26). Theories on the mechanism by which these compounds inhibit the formation of the initiation complex include: 1) a reduced ability of mRNA which has incorporated drug to bind to the initiation complex or 2) inhibition of the processing of nuclear heterogeneous RNA to mature cytoplasmic mRNA resulting in less mRNA being available to bind with the complexes. Both of these mechanisms may contribute to the inhibition of protein synthesis caused by this class of RNA "incorporators."

The results presented here demonstrate that the exponential type of cell lethality produced by some nucleoside analogs correlates with their ability to cause an accumulation of the 45 S rRNA initial transcript in the nucleolus. Three compounds tested, toyocamycin, 5-fluorouracil, and tubercidin, produced an accumulation of the 45 S precursor to rRNA and caused multiple logs of cell kill within the same concentration range. Concurrent inhibition of protein synthesis by tubercidin did not alter the exponential lethal effects. In contrast, sangivamycin, 8-azaguanine, and 5-azacytidine, which did not cause an accumulation of the 45 S rRNA precursor, produced a gradual and self-limiting type of cell lethality. 5-Azacytidine inhibits protein synthesis at a lower concentration than that which causes the accumulation of 32 S rRNA. Therefore, the two effects do not appear to be related. It is interesting that 5-azacytidine, which inhibits the processing of 32 S rRNA but does not accumulate 45 S rRNA, does not produce an exponential type of cell kill. Therefore, inhibition of rRNA processing without accumulation of 45 S rRNA precursor is not sufficient to cause exponential cell kill. Further characterization on the molecular level of the changes in RNA caused by these two classes of compounds may provide information on how RNA structure relates to function.

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