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9-Deazaadenosine

Cytocidal Activity and Effects on Nucleic Acids and Protein Synthesis in Human Colon Carcinoma Cells in Culture

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SUMMARY

The effect of 9-deazaadenosine (c^9Ado) on cell lethality and the synthesis of nucleic acids was investigated in human colon carcinoma cell line HT-29. c^9Ado produced a rapid threshold-exponential reduction in colony formation as measured by a soft agar clonogenic assay. This effect was evident after either a 2- or 24-hr exposure interval, and was produced over a very narrow concentration range of drug. Following 2 hr of drug exposure at concentrations producing a 1- to 3-log reduction in cell viability, DNA and RNA syntheses were inhibited 20% and protein synthesis was inhibited 35–50%. The latter effect became quite pronounced in comparison to nucleic acid synthesis 4 hr after drug treatment. Long treatment intervals (24 hr) with concentrations of c^9Ado producing similar effects on cell viability resulted in 15–35% inhibition of RNA synthesis, 80–85% inhibition of DNA synthesis, and 60–70% inhibition of protein synthesis. None of these metabolic effects could be accounted for by changes in ribonucleoside triphosphate levels despite the considerable formation of c^9ATP. Measurements of the incorporation of [^{3}H]c^9Ado into total cellular nucleic acids indicated that the labeling of RNA was 40–80% greater than that of DNA. Polysomal poly(A)RNA contained 300% more [^{3}H]c^9Ado than non-poly(A)RNA after 2 hr of drug exposure and 50% more [^{3}H]c^9Ado following 24 hr of treatment. There was no evidence of DNA strand breakage by incorporated c^9Ado. Analysis of nascent protein synthesis in drug-treated cells revealed that this process was inhibited in concert with polysome breakdown. These results suggest that the rapidity by which cell lethality is produced by c^9Ado may be related to inhibition of translation via its incorporation into RNA.

INTRODUCTION

Among the adenosine analogues resistant to adenosine deaminase, the newly synthesized pyrrolopyrimidine, c^9Ado,[1] has been found to be the most cytotoxic nucleoside yet discovered against murine and human leukemia cell lines *in vitro* (1). In preliminary reports, c^9Ado was a potent growth inhibitor of nine human solid tumors *in vitro* and possessed antitumor activity against a human pancreatic carcinoma xenograft (2). Thus far, there has been no systematic investigation of the mechanism of action of c^9Ado nor of its lethal effects against human tumors. In the present paper, we report the effects of this analogue on nucleic acid metabolism, protein synthesis, and cell lethality in a human colon carcinoma in culture.

[1] The abbreviations used are: c^9Ado, 9-deazaadenosine; PBS, phosphate-buffered saline (6.3 mM Na_2HPO_4, 0.8 mM KH_2PO_4, 0.154 M NaCl, pH 7.4); c^9ATP, 9-deazaadenosine 5′-triphosphate; HPLC, high-pressure liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials. [5-^{3}H]Urd (26.4 Ci/mmole), [U-^{14}C]Urd (582 mCi/mmole), [*methyl*-^{14}C]dThd (53.4 mCi/mmole), and [^{35}S]L-methionine (1053 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). [G-^{3}H]c^9Ado (18 Ci/mmole) was obtained from Moravek Biochemicals (Brea, Calif.). c^9Ado was generously provided by Drs. Robert S. Klein and Mu-Ill Lim, Sloan-Kettering Institute (New York, N. Y.).

Tissue culture. HT-29 cells were grown under 5% CO_2/air in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and gentamicin, 50 μg/ml. Cell inocula consisted of 0.83×10^5 cells/10 ml of medium in 25-cm^2 plastic flasks (Costar, Cambridge, Mass.) or increased 10-fold in 150-cm^2 flasks.

Drug treatment. Cells were incubated with the indicated concentrations of c^9Ado for 2 or 24 hr. Following drug exposure, cells were harvested by trypsinization (3).

Cell viability determinations. Soft agar cloning was performed as previously described (3). Cell viability is expressed as the number of surviving colonies from drug-treated cells + the number of colonies from control cells (corrected for cloning efficiency) × 100. Cloning efficiency ranged from 60% to 80%.

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DNA, RNA, and protein syntheses. Following addition of c^{9}Ado, cells grown in 25-cm^2 flasks were pulse-labeled with 0.5 μCi of [^{14}C]dThd and 5 μCi of [^{3}H]Urd or separately with 10 μCi of [^{35}S]L-methionine during the last hour of treatment. Cells were harvested by trypsinization, washed twice with 10 ml of ice-cold PBS and collected on glass-fiber filter discs after precipitation with either cold 10% trichloroacetic acid for determination of DNA and RNA synthesis or hot (90° for 10 min) 10% trichloroacetic acid for the determination of protein synthesis. Results are expressed as disintegrations per minute incorporated per 10^6 cells.

Polysomal non-poly(A)- and poly(A)RNA. Polysomes were isolated from HT-29 cells grown in duplicate 150-cm^2 flasks by the Mg^{2+} precipitation method previously described (4). RNA was removed by sodium dodecyl sulfate/phenol/chloroform extraction and separated into non-poly(A)- and poly(A)RNA by poly(U)Sepharose (Pharmacia Fine Chemicals, Piscataway, N. J.) chromatography (4). Agarose-urea gel electrophoresis was performed as previously described (3).

Drug incorporation into RNA and DNA. Incorporation of [^{3}H]c^{9}Ado into RNA and DNA was carried out as previously described (3). Cells were treated for 2 hr with 2.5×10^{-6} M (900 dpm/pmole) or 5×10^{-6} M (450 dpm/pmmole) [^{3}H]c^{9}Ado or for 24 hr with 2.5×10^{-7} M (9000 dpm/pmole) or 5×10^{-7} M (4500 dpm/pmole) [^{3}H]c^{9}Ado.

Nucleotide analyses. Ribonucleoside triphosphates were measured in neutralized 5% tricholoracetic acid extracts by anion-exchange HPLC (5) using isocratic elution with 0.36 M KH_2PO_4 (pH 3.8)/5% (v/v) acetonitrile. The retention times (minutes) for c^{9}ATP, CTP, UTP, ATP, and GTP were 3.7, 4.2, 4.8, 6.3, and 8.5.

Alkaline agarose gel electrophoresis. DNA strand breakage was measured in alkaline 0.7% agarose gels by the method of Freidenrich and Hand (6). DNA was extracted as described previously (3) from cells grown in 75-cm^2 flasks and prelabeled with [^{14}C]dThd, dissolved in 75 μl of formamide and heated at 100° for 5 min before its application to the gel.

Nascent protein synthesis. Cells grown in 150-cm^2 flasks were prelabeled with 10 μCi of [^{3}H]Urd for 2 days followed by incubation of cells in nonradioactive medium for 1 day. After incubation with 2.5×10^{-6}, 5×10^{-6}, or 1×10^{-5} M c^{9}Ado for 2 hr, cells were trypsinized and washed once in 10 ml of the methionine-free RPMI 1640 medium. Cells were resuspended in 0.5 ml of methionine-free RPMI 1640 medium with 10% fetal calf serum, equilibrated at 37° for 5 min, and incubated with 100 μCi of [^{35}S]methionine for 2 min. Incubations were stopped by the addition of 2.5 ml of ice-cold PBS and centrifugation at 400 × *g* for 5 min at 4°. The cell pellet was homogenized in 0.5 ml of PBS containing 1% NP-40, and the homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was layered onto a 10–30% glycerol gradient containing 20 mM 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (pH 7.5)/75 mM KCl/2.5 mM magnesium acetate and centrifuged at 39,000 rpm at 4° for 4 hr in an SW 41 rotor (7) to resolve the 80 S monosome and 40 S subunit. Fractions were collected from the bottom of each gradient and collected on glass-fiber filter discs by precipitation with 10% trichloroacetic acid.

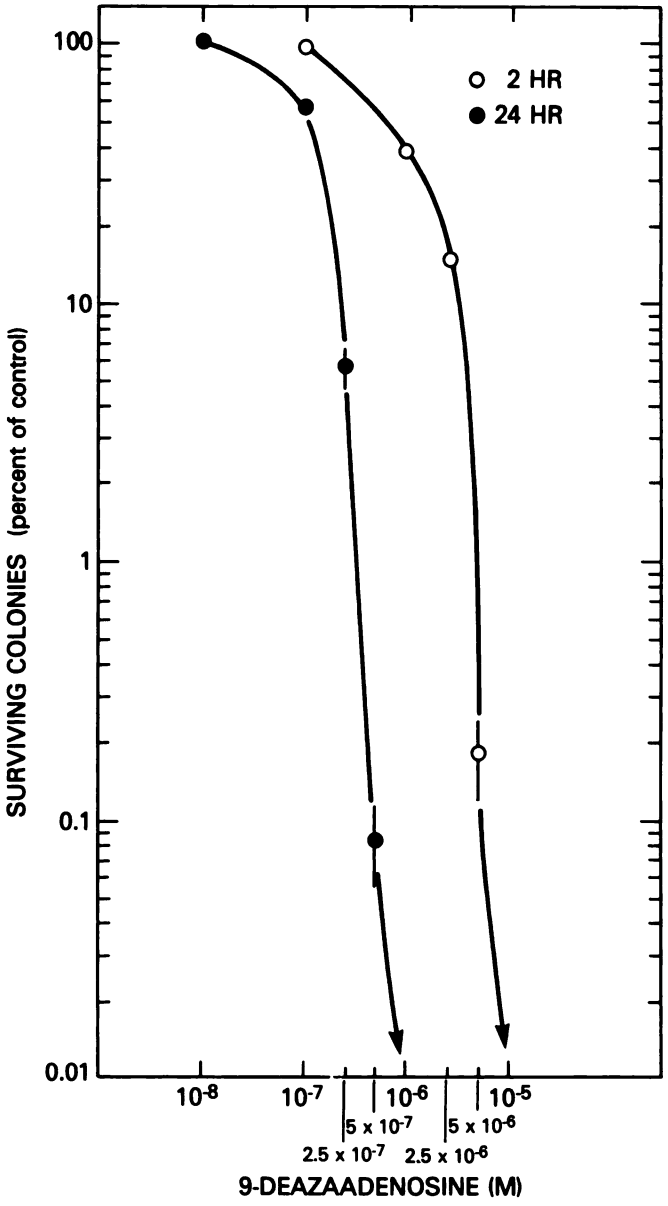

FIG. 1. *Viability of HT-29 cells following exposure to c^{9}Ado*
Cells were exposed to c^{9}Ado for 2 or 24 hr, and cell viability was determined by the soft agar clonogenic assay described under Experimental Procedures. Results are expressed as the percentage of surviving colonies *versus* control. Each value represents the mean ± standard error of four to seven duplicate experiments.

RESULTS

Cell viability. Soft agar clonogenic assays were used to determine the effect of short-term (2 hr) and long-term (24 hr) treatment of HT-29 cells with c^{9}Ado (Fig. 1). Both exposure intervals produced a threshold-exponential type of cell kill which possessed identical characteristics of concentration times time. Most noticeable was the sharp reduction in colony formation over a very narrow concentration range at both drug-exposure intervals, an effect which is highly unusual for nucleoside analogues with this cell line.

DNA, RNA, and protein synthesis. Under conditions identical with those used for determining cell viability, HT-29 cells were pulse-labeled with [^{14}C]dThd, [^{3}H]Urd, and [^{35}S]methionine as an approximate measure of DNA, RNA, and protein synthesis, respectively (Fig. 2). Following 2 hr of drug exposure, DNA and RNA syntheses were inhibited by only 20% at drug concentrations producing 1- and 3-log reductions in colony formation; protein synthesis was inhibited by 35–50% under these conditions. Twenty-four hour treatment with c^{9}Ado produced a 15–35% inhibition of RNA synthesis, 80–85% inhibition of DNA synthesis, and 60–70% inhibition of protein synthesis at similar cytocidal concentrations of drug.

A time course of the effect of c^{9}Ado on [^{3}H]Urd and [^{35}S]Met incorporation into cold trichloroacetic acid- and hot tricholoracetic acid-precipitable radioactivity, respectively, is shown in Fig. 3. At both 5×10^{-6} and 1×10^{-5} M c^{9}Ado, protein synthesis abruptly decreased after

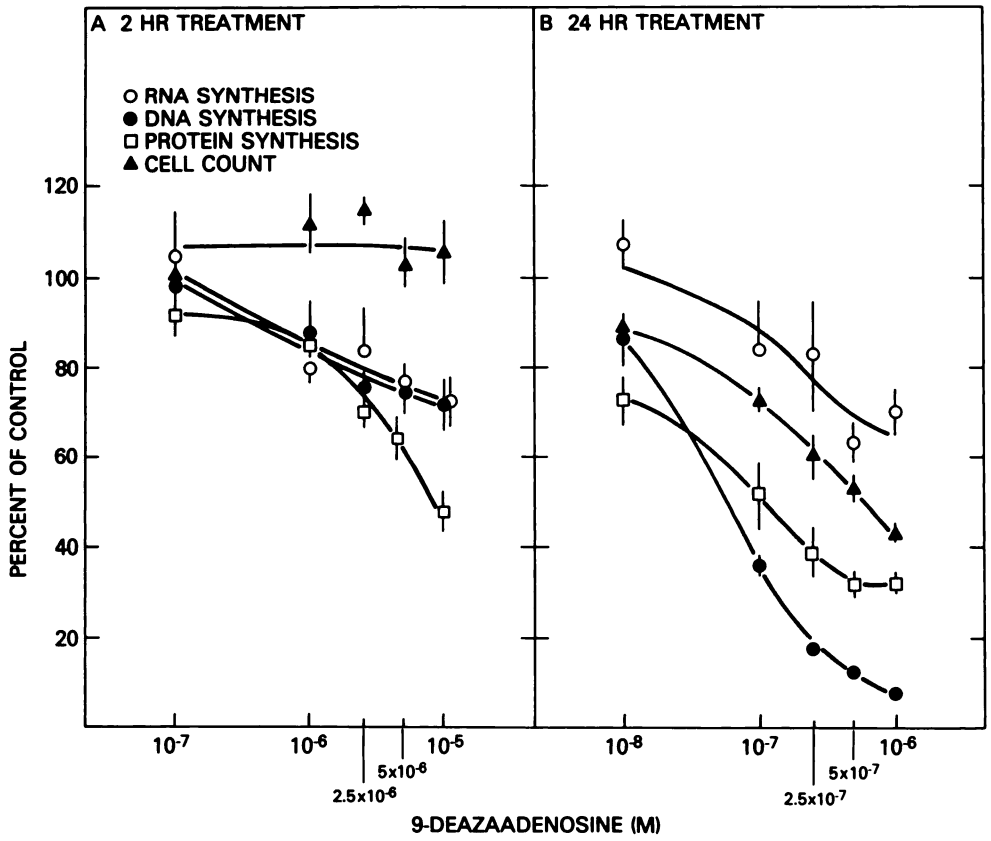

FIG. 2. *Incorporation of radiolabeled precursors into DNA, RNA, and protein following exposure to c⁹Ado*

Cells were treated with c⁹Ado for 2 hr (A) or 24 hr (B) and pulse-labeled with [¹⁴C]dThd, [³H]Urd, or [³⁵S]methionine during the last hour of treatment. Incorporation of each precursor is expressed as a percentage of control values (disintegrations per minute/10⁶ cells) which were as follows: DNA, 20,000–40,000; RNA, 50,000–80,000; and protein, 25,000–50,000. Each value represents the mean ± standard error of six to eight experiments.

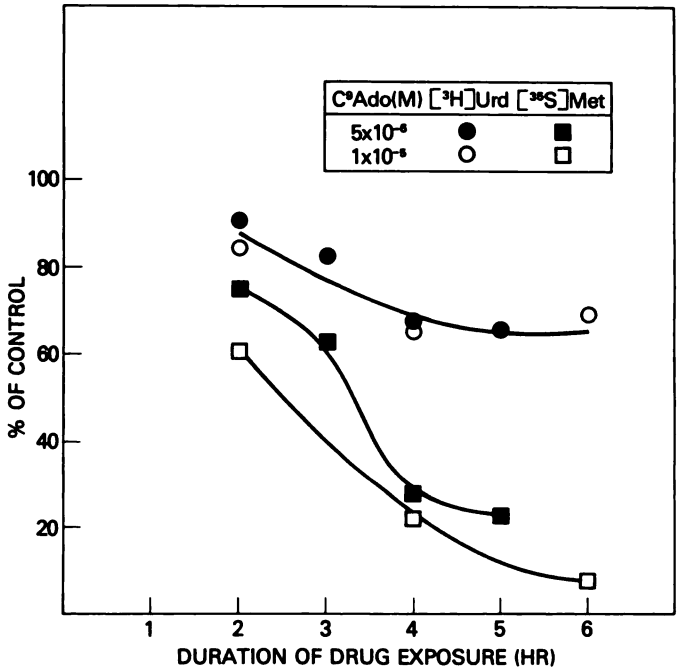

FIG. 3. *Incorporation of [³H]Urd and [³⁵S]Met into RNA and protein following varying durations of exposure to c⁹Ado*

Cells were treated with 5×10^{-6} M c⁹Ado (●, ■) for 2, 3, 4, and 5 hr and labeled during the last hour of incubation with [³H]Urd (●, ○) and [³⁵S]Met (□, ■). Cells were similarly treated with 1×10^{-5} M c⁹Ado (○, □) for 2, 4, and 6 hr. Incorporation of precursors is expressed as a percentage of control values. Each value represents the mean of triplicate samples.

4 hr of drug treatment, whereas RNA synthesis was inhibited by 10–35% throughout the incubation periods. A striking dichotomy between the two processes was obvious by 4–6 hr after exposure to c⁹Ado.

To assess whether c⁹Ado preferentially affected the synthesis of specific species of RNA, polysomal RNA was isolated and fractionated into non-poly(A)RNA (predominately rRNA and tRNA) and poly(A)RNA (mRNA) (Table 1). Treatment of cells for 2 hr with c⁹Ado did not significantly affect the synthesis of polysomal RNA.

Since inhibition of protein synthesis appeared to be a major initial effect of c⁹Ado, nascent peptide synthesis was further evaluated by prelabeling ribosomes with [³H]Urd followed by rapidly pulse-labeling the cells with [³⁵S]methionine for 2 min. Glycerol gradient centrifugation was subsequently utilized to resolve the 80 S monosome and 40 S ribosomal subunit (Fig. 4). Control cells pulsed with [³⁵S]methionine incorporated most of the precursor into nascent proteins which migrated to the top of the gradient with tRNA (Fractions 19–26, Fig. 4A), while a small amount of labeled protein coincided with the 40 S subunit, presumably as an initiation complex (Fractions 11–14, Fig. 4A). Treatment of cells for 2 hr with c⁹Ado

TABLE 1

Ribonucleoside triphosphate and c⁹ATP levels in HT-29 cells following treatment with c⁹Ado

Cells were treated for 2 or 24 hr with c⁹Ado, and nucleotide concentrations were determined by anion-exchange HPLC as described under Experimental Procedures. Each value represents the mean ± standard error of four or five experiments.

Treatment	CTP	UTP	ATP	GTP	c⁹ATP
		nmoles/10⁶ cells			*nmoles/10⁶ cells*
Control, 2 hr	2.31 ± 0.13	6.26 ± 0.70	11.94 ± 1.08	3.07 ± 3.4	
			% of control		
c⁹Ado, 2 hr					
1 × 10⁻⁶ M	88 ± 6	78 ± 4	83 ± 6	87 ± 9	0.55 ± 0.20
2.5 × 10⁻⁶ M	112 ± 13	88 ± 9	96 ± 10	98 ± 8	1.03 ± 0.23
5 × 10⁻⁶ M	139 ± 16	102 ± 6	111 ± 12	128 ± 14	2.35 ± 0.55
1 × 10⁻⁵ M	129 ± 15	79 ± 8	76 ± 9	112 ± 14	5.31 ± 1.03
			nmoles/10⁶ cells		
Control, 24 hr	1.56 ± 0.09	3.16 ± 0.39	8.50 ± 0.39	2.18 ± 0.15	
			% of control		
c⁹Ado, 24 hr					
1 × 10⁻⁷ M	143 ± 12[a]	121 ± 4	138 ± 3[c]	126 ± 4[a]	0.24 ± 0.10
2.5 × 10⁻⁷ M	173 ± 25[a]	122 ± 2	158 ± 15[a]	144 ± 19	0.43 ± 0.10
5 × 10⁻⁷ M	220 ± 16[c]	137 ± 11	184 ± 7[c]	177 ± 9[b]	0.90 ± 0.43
1 × 10⁻⁶ M	296 ± 23[c]	144 ± 9[a]	173 ± 13[b]	208 ± 13[c]	3.39 ± 1.02

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

resulted in inhibition of nascent protein synthesis and in [^{35}S]methionine-associated radioactivity with the 40 S subunit (Fig. 4B–E) to a magnitude comparable to that of total protein synthesis measured after 1 hr (Fig. 2). Most striking was the increase in formation of 80 S monosome after drug treatment, even after exposure to 1 × 10⁶ M c⁹Ado, where protein synthesis was inhibited by only 15% (Fig. 4B).

Nucleotide analysis. To determine whether c⁹Ado was metabolized to the nucleoside triphosphate and whether alterations in the nucleotide levels of treated cells were related to cytotoxicity, anion-exchange HPLC of cell extracts was performed (Fig. 5; Table 1). A representative HPLC profile after 2 hr of drug exposure revealed that a metabolite of c⁹Ado with a retention time of the analogue triphosphate eluted just before CTP (Fig. 5). The for-

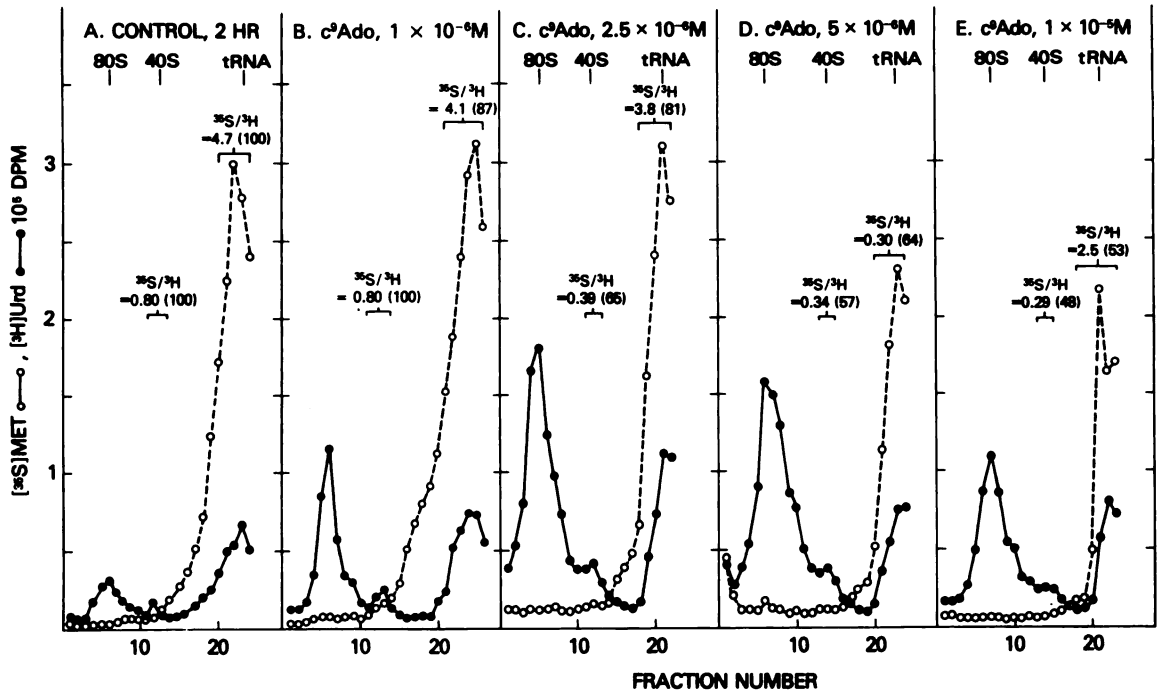

FIG. 4. *Initiation of protein synthesis in HT-29 cells treated with c⁹Ado*

Cells were prelabeled with 10 μCi of [^{3}H]Urd (●) and pulse-labeled for 2 min with 100 μCi of [^{35}S]methionine (○). The 10,000 × *g* supernatant from control and c⁹Ado-treated cells was resolved in 10–30% glycerol gradients as described under Experimental Procedures. The direction of sedimentation is from *right* to *left*.

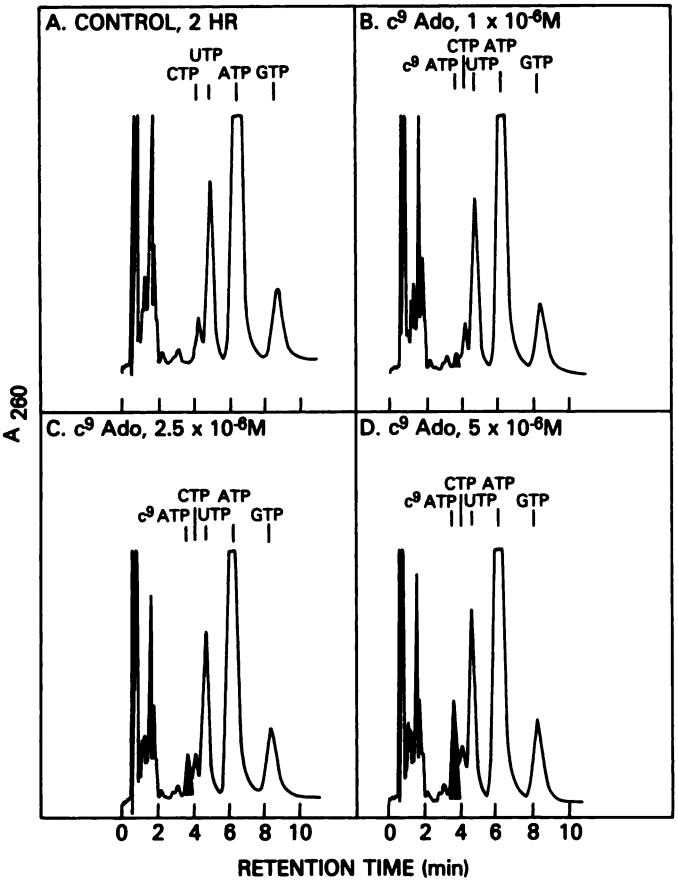

FIG. 5. *HPLC of nucleoside triphosphates in HT-29 cells treated with c⁹Ado*

Cells were treated with c⁹Ado for 2 hr, and cellular tricholoracetic acid extracts were chromatographed by anion-exchange HPLC as described under Experimental Procedures.

mation of c⁹ATP was concentration- and time-dependent, but no significant alterations in nucleotide levels occurred after 2 hr of drug treatment (Table 2). The longer explosure interval, however, resulted in moderate elevations of virtually all nucleoside triphosphates.

Incorporation of [³H]c⁹Ado into RNA and DNA. The metabolism of c⁹Ado to the triphosphate suggested that it may serve as a precursor for RNA and DNA. Consequently, experiments were designed to measure the incorporation of [³H]c⁹Ado into total cellular RNA and DNA (Fig. 6). At concentrations producing 1-log and 3-log reductions in colony formation, [³H]c⁹Ado was incorporated into RNA, and into DNA after 2 and 24 hr of drug exposure. The incorporation of drug into RNA was approximately 40–80% greater than that into DNA.

Studies were next carried out to determine whether [³H]c⁹Ado was preferentially incorporated into non-poly(A)- or poly(A)RNA from polysomes. Exposure of cells for 2 or 24 hr to [³H]c⁹Ado resulted in a greater incorporation of drug into poly(A)RNA than into non-poly(A)RNA (Table 3). Although there was proportionally more drug incorporated into both RNA fractions upon long-term drug treatment based on concentration times time considerations, the ratio of drug incorporated into poly(A)RNA versus non-poly(A)RNA was twice as great after short-term drug exposure. Electrophoretic analysis of polysomal RNA labeled with [³H]c⁹Ado for 2 hr (Fig. 7A and B) or 24 hr (Fig. 7C and D) revealed that non-poly(A)RNA consisted primarily of rRNA and tRNA whereas poly(A)RNA possessed its characteristic heterodisperse pattern. There was no manifestation of aberrant processing of rRNA by treatment with 2.5×10^{-6} M c⁹Ado for 2 hr (Fig. 7A) or with 2.5×10^{-7} M c⁹Ado for 24 hr (Fig. 7C).

Since c⁹Ado was also incorporated into DNA, although to a lesser extent than into RNA, alkaline agarose electrophoresis of DNA isolated from drug-treated cells was performed. No differences were observed in alkali-denatured, single-stranded DNA between control and c⁹Ado-treated cells after exposure for either 2 or 24 hr to concentrations of c⁹Ado used in the experiments shown in Fig. 6 (results not shown). Thus, no single-stranded breaks in DNA were produced as a result of drug substitution.

TABLE 2

Effect of c⁹Ado on polysomal RNA

Cells were treated with 2.5×10^{-6} M or 5×10^{-6} M c⁹Ado for 2 hr and labeled with 10 μCi of [¹⁴C]Urd during the last hour of incubation. Polysomal RNA was extracted and fractionated on poly(U)Sepharose as described under Experimental Procedures. Results represent the mean ± standard error of three experiments and are expressed as a percentage of control values.

Treatment	Non-poly(A)RNA	Poly(A)RNA
	% of Control	
c⁹Ado		
2.5×10^{-6} M	98 ± 9	132 ± 16
5×10^{-6} M	93 ± 7	118 ± 18

DISCUSSION

The new antitumor C-nucleoside, c⁹Ado, has previously been reported to possess an IC_{50} in the nanomolar range upon continuous exposure of murine and human leukemia cell lines to drug *in vitro* (1). The present investigation confirms the high potency of this analogue in a human colon carcinoma, and in fact this drug appears to be the most potent nucleoside analogue in this cell line. Of particular note was the rapid exponential cell kill over a very narrow concentration range of drug. In comparison to other antitumor agents in the cell line, c⁹Ado was equally as potent as 5-fluorouridine (3) and about one-tenth as potent as Adriamycin (8) upon 2 hr of drug treatment; however, after 24 hr of drug exposure, c⁹Ado was 10-fold more cytocidal than 5-fluorouridine and equally as potent as Adriamycin. Unlike the latter two drugs, c⁹Ado produces a 100-fold increase in cell lethality by only a doubling of its concentration.

Recent studies by Deeprose *et al.* (9) revealed that cytolytic lymphocytes were much less sensitive to c⁹Ado upon 1–3 hr of drug exposure than after 22 hr of treatment. This effect is related to the slow phosphorylation of c⁹Ado to c⁹ATP and a fall in ATP. From the present study, it is clear that HT-29 cells rapidly metabolize c⁹Ado to the triphosphate without ATP depletion. Moreover, there is no relationship between cell lethality and depletion of any ribonucleoside triphosphate.

Although c⁹Ado was incorporated into both RNA and

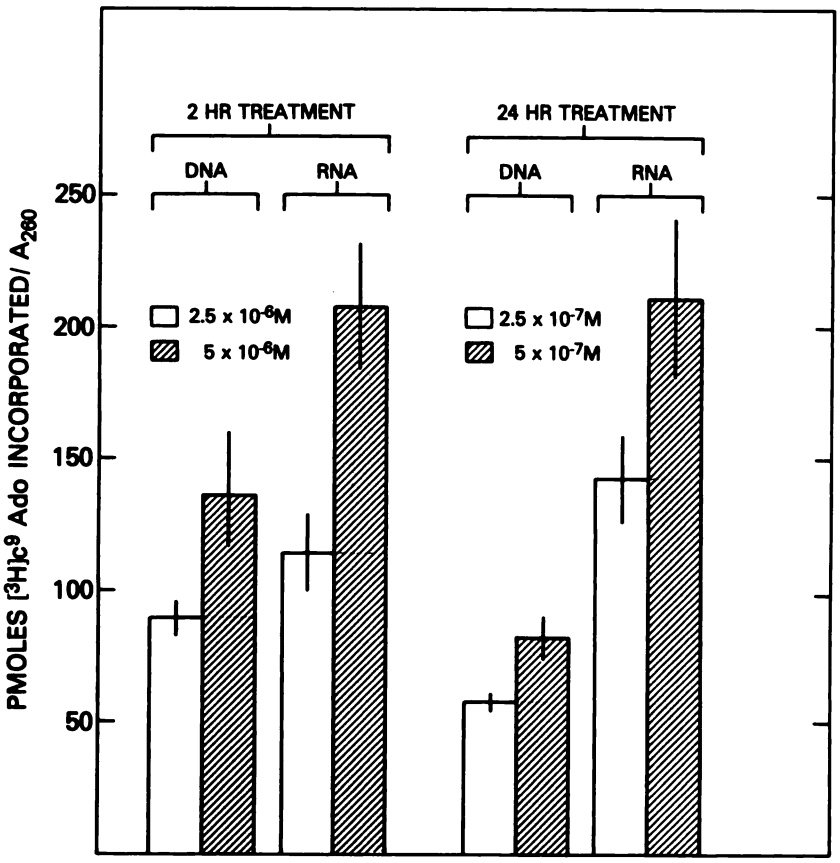

FIG. 6. *Incorporation of [^{3}H]c^{9}Ado into total RNA and DNA*

Cells were treated for 2 or 24 hr with [^{3}H]c^{9}Ado, and DNA and RNA were isolated as described under Experimental Procedures. Each value is the mean ± standard error of three experiments.

DNA, DNA strand breakage was not produced and polysomal RNA synthesis was equally unaffected. There was also no evidence of interference with the methylation of polysomal RNA (results not shown) nor of the processing of RNA. This contrasts with another pyrrolopyrimidine analogue, 7-deazaadenosine (tubercidin), which has been reported to impair markedly the processing of ribosomal precursor RNA to 28 S and 18 S RNA (10).

On the other hand, large amounts of drug were incorporated into rRNA, tRNA, and poly(A)RNA, which could have affected translation. The early temporal reduction in protein synthesis in comparison to a more moderate and lesser inhibition of RNA synthesis indicates that inhibition of translation is related to the rapid onset of cell lethality. The enhanced formation of 80 S monosomes coincident with this effect suggests that initiation of protein synthesis was affected by c^{9}Ado (11). However, no evidence of impaired translational activity of mRNA from c^{9}Ado-treated cells was obtained *in vitro* using a rabbit reticulocyte lysate system[2]; hence, these data suggest that drug-modified mRNA is not likely to account for inhibition of translation. However, drug modification of rRNA or tRNA still remains a viable possibility. Previous studies with tubercidin revealed that 3′-substitution of tRNA did not markedly affect aminoacylation (12), and could not account for the preferential inhibition of protein synthesis produced by this drug (13, 14). However, it is possible that the initiator codon A-U-G or perhaps another binding site in tRNA is modified by c^{9}Ado or tubercidin. Alternatively, drug-modified rRNA could also result in altered binding sites in the 40 S subunit for mRNA.

A second possibility is that c^{9}Ado competes with ATP for aminoacylation of tRNA or perhaps with GTP in translocation and elongation reactions. Such inhibitory effects would be expected to be competitive, as was found for tubercidin 5′-triphosphate (12). Since the ratio of ATP/c^{9}ATP was 6:1 for a 2-hr exposure to 5×10^{-6} M

TABLE 3

Incorporation of [^{3}H]c^{9}Ado into polysomal RNA of HT-29 cells

Cells were treated with [^{3}H]c^{9}Ado for either 2 or 24 hr, and polysomal RNA was extracted and fractionated on poly(U)Sepharose as described under Experimental Procedures. Each value represents the mean ± standard error of three experiments.

Treatment	Polysomal RNA		Ratio poly(A)RNA/ Non-poly(A)RNA
	Non-poly(A)RNA	Poly(A)RNA	
	pmoles c^{9}Ado incoporated/A_{260}		
[^{3}H]c^{9}Ado			
2 hr			
2.5×10^{-6} M	43 ± 5	117 ± 17	2.72
5×10^{-6} M	59 ± 11	193 ± 31	3.27
24 hr			
2.5×10^{-7} M	141 ± 14	175 ± 17	1.24
5×10^{-7} M	212 ± 12	397 ± 15	1.87

[2] R. I. Glazer and K. D. Hartman, unpublished results.

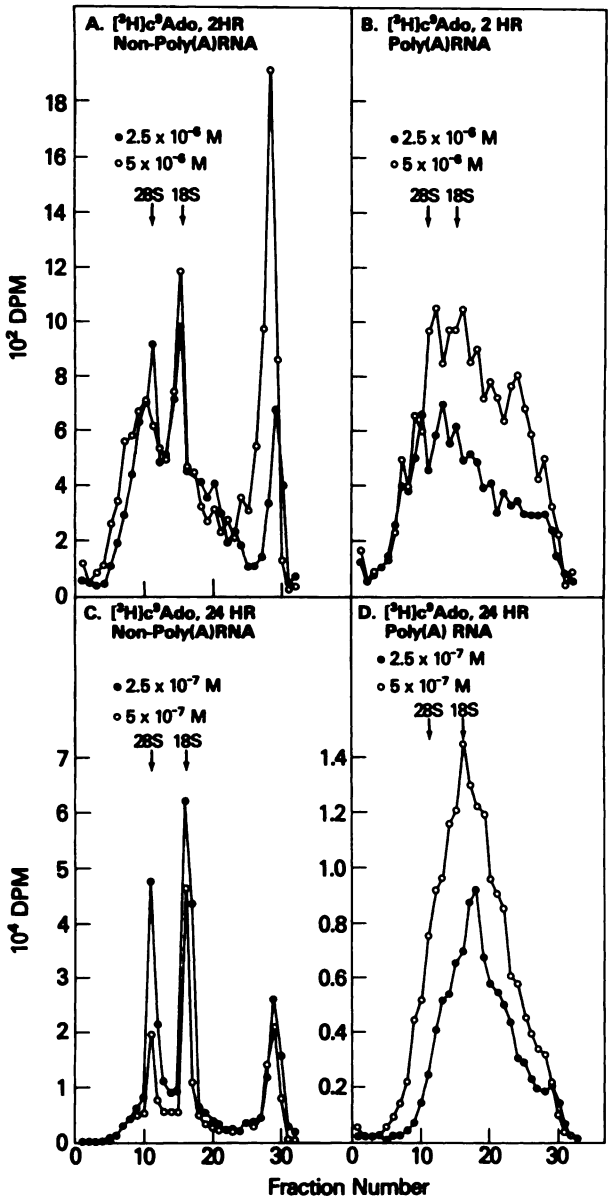

FIG. 7. *Agarose gel electrophoresis of polysomal RNA labeled with [^{3}H]c^{9}Ado*

Cells were labeled for 2 or 24 hr with [^{3}H]c^{9}Ado, and polysomal RNA was prepared and fractionated on poly(U)Sepharose as described under Experimental Procedures.

c^{9}Ado (Table 1), a condition resulting in 40% inhibition of protein synthesis, and 10:1 for a 24-hr treatment with 5×10^{-7} M c^{9}Ado, where 70% inhibition of protein synthesis occurred (Table 1; Fig. 2), it is not likely that the level of c^{9}ATP competitively inhibited translation. Similarly, the concentration of GTP relative to c^{9}ATP was not related to the progressively greater inhibition of protein synthesis observed with increasing exposure interval and drug concentrations.

Thus, the experimental evidence suggests that c^{9}Ado primarily interferes with protein synthesis, probably at the level of tRNA and/or rRNA. This effect in turn results in inhibition of initiation of translation coincident with a resultant loss in cell viability.

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