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In Vitro Translational Activity of Messenger RNA following Treatment of Human Colon Carcinoma Cells with Sangivamycin

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SUMMARY

Total mRNA from human colon carcinoma cell line HT-29 treated with the pyrrolopyrimidine antibiotic, sangivamycin (7-deaza-7-carboxamidoadenosine), was assessed *in vitro* using a reticulocyte lysate translation system. Under conditions of known drug-induced cell lethality, sangivamycin-modified mRNA showed a diminished translational capacity. The decreased activity of drug-modified mRNA increased in proportion to the time-dependent cytocidal effects of sangivamycin in this cell line. These data suggest that the lethal effects of this drug may be associated, in part, with a reduced ability of drug-modified mRNA to sustain translation *in situ*.

INTRODUCTION

The pyrrolopyrimidine antibiotic, sangivamycin,[1] has been previously shown to possess significant time-dependent cytocidal activity against mouse sarcoma 180 and human colon carcinoma HT-29 *in vitro* (1, 2). Associated biochemical effects produced by the drug include inhibition of the synthesis of DNA (1, 2) as well as of rRNA and poly(A)-containing polysomal RNA with little or no effect on RNA methylation (3, 4). Recently, we reported that sangivamycin is incorporated preferentially into RNA of sarcoma 180 cells (5) and that the time-dependent incorporation of drug into total poly(A)-containing RNA in sarcoma 180 and HT-29 cells appeared to show a significant correlation with the lethal effects of the drug (2, 5).

In order to understand the functional behavior of drug-modified mRNA and to determine whether this effect may be causally associated with the cytocidal properties of sangivamycin, we undertook experiments wherein the total mRNA of drug-treated HT-29 cells was translated in a cell-free system *in vitro* under conditions of known drug toxicity.

EXPERIMENTAL PROCEDURES

Materials. L-[^{35}S]Methionine (1053 Ci/mmole), [5-^{3}H]Urd (25 Ci/mmole), and reticulocyte lysate translation kits were purchased from New England Nuclear Corporation (Boston, Mass.). Sangivamycin was obtained from the Natural Products Branch, National Cancer Institute (Bethesda, Md.).

Cell culture. HT-29 cells were initially plated at a density of 8.3×10^5 cells/100 ml of medium and grown in 150-cm^2 plastic flasks (Costar) under an atmosphere of 5% CO_2/air in RPMI medium 1640 supplemented with 10% heat-inactivated fetal calf serum (GIBCO) and gentamicin, 50 μg/ml. Logarithmically growing (3-day) cells were incubated with 1×10^{-6} M sangivamycin for 24 or 48 hr. RNA was pulse-labeled with 100 μCi of [5^{-3}H]Urd/100 ml of medium for 1 hr before the cells were harvested.

RNA isolation. A modification of the procedure of Strohman *et al.* (6) and Patterson and Bishop (7) was used. Cells were rinsed with 10 ml of ice-cold Hanks' balanced salt solution and immediately scraped into 6 ml of −20° 8 M guanidine (pH 7)/2 M potassium acetate (pH 5)(19:1, v/v). Two 150-cm^2 flasks were used per 6 ml of guanidine/acetate buffer. The cell lysate, maintained at 4°, was homogenized with 15 strokes of a motor-driven Teflon-glass homogenizer driven at full speed. The homogenate was mixed with 0.5 volume of 95% ethanol and precipitated for 2 hr at −20°. The samples were centrifuged at 8,000 × *g* for 10 min at 4° and drained; the pellet was dissolved in 4.75 ml of 8 M guanidine (pH 7) and 0.25 ml of 0.5 M EDTA (pH 7). RNA was precipitated with 0.5 volume of 2% potassium acetate in 95% ethanol for 2–4 hr at −20°. Samples were centrifuged at 8,000 × *g* for 10 min at 4°, dissolved in 1 ml of 0.02 M EDTA (pH 7), and mixed with 3 ml of chloroform/1-butanol (4:1). Phases were separated at 3,000 × *g* for 1 min; the upper phase was saved and the interphase was reextracted with 2 ml of 0.02 M EDTA. The two aqueous phases were combined and precipitated with 2 volumes of 4.5 M sodium acetate (pH 6) overnight at −20°. Samples were centrifuged at 12,000 × *g* for 30 min at 4° and drained; the RNA precipitate was dissolved in 1 ml of water and reprecipitated with 3 volumes of 2% potassium acetate in 95% ethanol for 2 hr at −20°. The RNA was centrifuged at 8,000 × *g* for 30 min, washed once with 95% ethanol, centrifuged at 8,000 × *g* for 15 min, drained, and dissolved in water. The A_{260}/A_{280} ratio ranged from 1.9 to 2.1.

Poly(U) Sepharose chromatography. Guanidine-extracted RNA was fractionated into non-poly(A)- and poly(A)RNA as previously described (2).

In vitro translation assay. RNA was translated in a reticulocyte lysate translation system. The assay contained, in a final volume of 25 μl, 10 μCi of [^{35}S]methionine, 10 μl of lysate, 0.7 mM magnesium acetate, 80 mM potassium acetate, 220 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4), 0.33 mM GTP, 0.22 mM spermidine, 1.1 mM creatine phosphate, 2.2 mM dithiothreitol, and 3–6 μg of total guanidine-extracted RNA or 0.5–2 μg of poly(A)RNA. Assay mixtures were

[1] The trivial name used is: sangivamycin, 7-deaza-7-carboxamidoadenosine.

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TABLE 1

Guanidine-extracted RNA from control and sangivamycin-treated cells

HT-29 cells were treated for 24 or 48 hr with 10^{-6} M sangivamycin, and RNA was extracted as described under Experimental Procedures. Each value is the mean ± standard error of six duplicate experiments.

Treatment	Exposure time	Guanidine-extracted RNA	
		Total A_{260}	A_{260}/A_{280}
	hr		
Control	24	12.9 ± 2.3 (100)	1.87 ± 0.02 (100)
Sangivamycin, 10^{-6} M	24	5.8 ± 1.3 (45)	1.87 ± 0.02 (100)
Control	48	14.0 ± 3.0 (100)	1.91 ± 0.02 (100)
Sangivamycin, 10^{-6} M	48	4.5 ± 1.2 (32)	1.91 ± 0.06 (100)

incubated at 37° for 1 hr. The reaction was stopped by the addition of 25 μl of 1 M Tris (pH 10.9), and the mixture was further incubated at 37° for 30 min to hydrolyze labeled tRNA. Aliquots (5 μl) were spotted on a strip of glass-fiber filter paper and chromatographed as previously described (8).

Electrophoresis. Duplicate translation assay samples (final volume of 100 μl) were dialyzed in Spectrapor tubing (6000–8000 mol wt cutoff) against 1 liter of electrophoresis sample buffer (2% sodium dodecyl sulfate/0.063 M Tris-HCl (pH 6.8)/10% glycerol/5 mM dithiothreitol) overnight at 4°. Five microliters of 1% bromophenol blue tracking dye were added after dialysis, and samples were electrophoresed in polyacrylamide slab gels (14 × 14 × 0.3 cm) containing a 5%, 1.5-cm high acylamide stacking gel above a 12.5-cm high 10% acrylamide separating gel (9).

Agarose gel electrophoresis. RNA was characterized by electrophoresis in 1.5% agarose/urea gels according to the method of Locker (10).

RESULTS

Previous studies of the effect of sangivamycin on cell lethality in HT-29 cells indicated that, although a 2-hr exposure to as much as 1×10^{-5} M sangivamycin was without effect, 24-hr or 48-hr exposure to 1×10^{-6} M drug produced 80% and 98% reductions, respectively, in colony formation (2). Thus, the present studies were initiated using identical treatment and growth conditions. Guanidine-extracted RNA from control and treated cells were found to be of similar purity as analyzed by the ratio of A_{260}/A_{280}, and recovery of total RNA was less in drug-treated cells, a result reflecting the lower cellularity under these conditions (Table 1). RNA was further characterized by agarose-urea gel electrophoresis, and a similar size distribution of [^{3}H]Urd-labeled RNA between control and sangivamycin-treated cells was noted (Fig. 1).

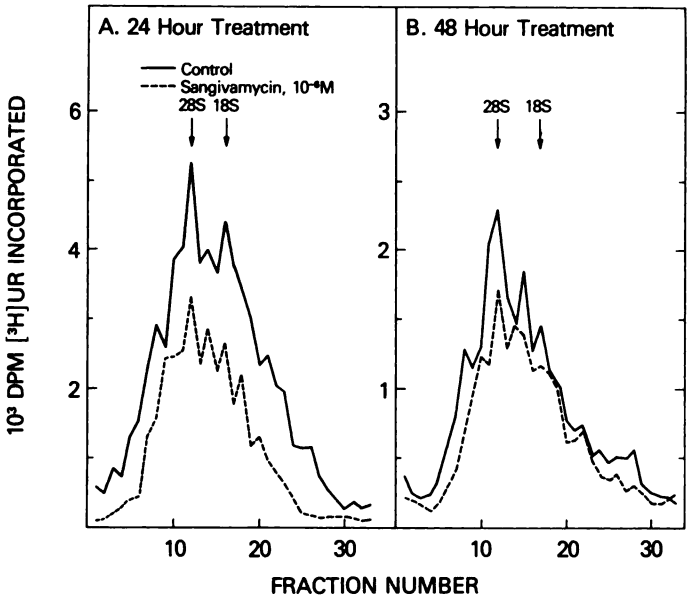

FIG. 1. *Agarose gel electrophoresis of guanidine-extracted RNA from HT-29 cells treated with sangivamycin*

Cells were treated for either 24 hr (A) or 48 hr (B) with 1×10^{-6} M sangivamycin and pulse-labeled during the last hr of treatment with [^{3}H]Urd as described under Experimental Procedures.

TABLE 2

RNA synthesis and in vitro translational activity in control and sangivamycin-treated cells

HT-29 cells were treated as described in Table 1 and pulse-labeled for the last hour with 100 μCi of [^{3}H]Urd. RNA was isolated and translated *in vitro* with [^{35}S]methionine as labeled precursor as described under Experimental Procedures. Each value is the mean ± standard error of five duplicate experiments.

Treatment	Exposure time	RNA synthesis	*In vitro* translational activity
	hr	*dpm/A_{260}/hr*	*10^5 dpm/μg RNA/hr*
Control	24	487,500 ± 53,200 (100)	1.20 ± 0.18 (100)
Sangivamycin, 10^{-6} M	24	357,300 ± 48,300 (73)	0.84 ± 0.07 (70)
Control	48	299,300 ± 24,500 (100)	1.11 ± 0.13 (100)
Sangivamycin, 10^{-6} M	48	187,800 ± 20,900 (63)[a]	0.57 ± 0.03 (51)[a]

[a] Statistically significant difference ($p < 0.01$) versus control values.

Analysis of the translational activity of RNA from sangivamycin-treated cells revealed that a time-dependent inhibition of translation occurred (Table 2). Reticulocyte lysate assays utilizing 6 μg of RNA from cells treated with 1×10^{-6} M sangivamycin for 24 or 48 hr showed a 29% and 48% inhibitions of translation activity, respectively, utilizing [^{35}S]methionine as the labeled precursor. The inhibitions of labeling of RNA by [^{3}H]Urd were 27% and 37% following 24- and 48-hr exposure to 10^{-6} M sangivamycin and were of a magnitude similar to previous results (2).

One possible explanation of the reduced translational activity of mRNA from drug-treated cells is that the mRNA content comprises a lesser percentage of the total

TABLE 3

Fractionation of guanidine-extracted RNA on poly(U) Sepharose

HT-29 cells were treated for 48 hr with 10^{-6} M sangivamycin, and RNA was extracted and fractionated by poly(U) Sepharose chromatography as described under Experimental Procedures. Each value is the mean ± standard error of six duplicate experiments.

Treatment	Non-poly(A)RNA	Poly(A)RNA	% Poly(A)RNA
	total A_{260} units		
Control	13.4 ± 2.5 (100)	1.6 ± 0.6 (100)	11
Sangivamycin, 10^{-6} M	5.3 ± 1.3 (40)	0.7 ± 0.3 (44)	12

TABLE 4

RNA synthesis and in vitro translational activity of guanidine RNA fractionated by poly(U) Sepharose chromatography

HT-29 cells were treated for 48 hr with 10^{-6} M sangivamycin, and RNA was pulse-labeled for the last hour with 100 μCi of [^{3}H]Urd. RNA was isolated and fractionated by poly(U) Sepharose chromatography as described under Experimental Procedures. Each value is the mean ± standard error of six duplicate experiments. Translation assays were carried out with 0.5 μg of poly(A)RNA, and values represent the mean ± standard error of three duplicate experiments.

Treatment	RNA synthesis		*In vitro* translational activity of poly(A)RNA
	Non-poly(A)RNA	Poly(A)RNA	
	dpm/A_{260}/hr		*10^6 dpm/μg RNA/hr*
Control	368,000 ± 29,000 (100)	647,400 ± 92,200 (100)	1.18 ± 0.08 (100)
Sangivamycin	217,400 ± 41,200 (59)	410,000 ± 66,200 (63)	0.71 ± 0.060 (60)[a]

[a] Statistically significant difference ($p < 0.01$) versus control values.

cellular RNA. To assess this possibility, RNA was fractionated by poly(U) Sepharose chromatography to obtain poly(A)RNA (mRNA) (Table 3). The proportions of poly(A)RNA were 11–12% in both control and drug-treated cells. Inhibition of non-poly(A)- and poly(A)RNA synthesis after treatment with sangivamycin for 48 hr was comparable to that of total RNA (Table 4). Similarly, reduced translational activity *in vitro* with poly(A)RNA from drug-treated cells was approximately equivalent to that found with total cellular RNA (Table 4).

An autoradiogram of the electrophoretic separation of the translation products produced by 3 μg and 6 μg of total RNA is shown in Fig. 2. The earliest and most prominent effect of the drug-modified mRNA was the absence of the 100,000 mol wt band and a reduced translation of proteins of 86,000, 73,000, and 47,000 mol wt following 24 hr of treatment. After 48 hr of drug treatment, virtually all of the translated products were reduced in concentration, and there was complete absence of the 100,000 and 86,000 mol wt proteins.

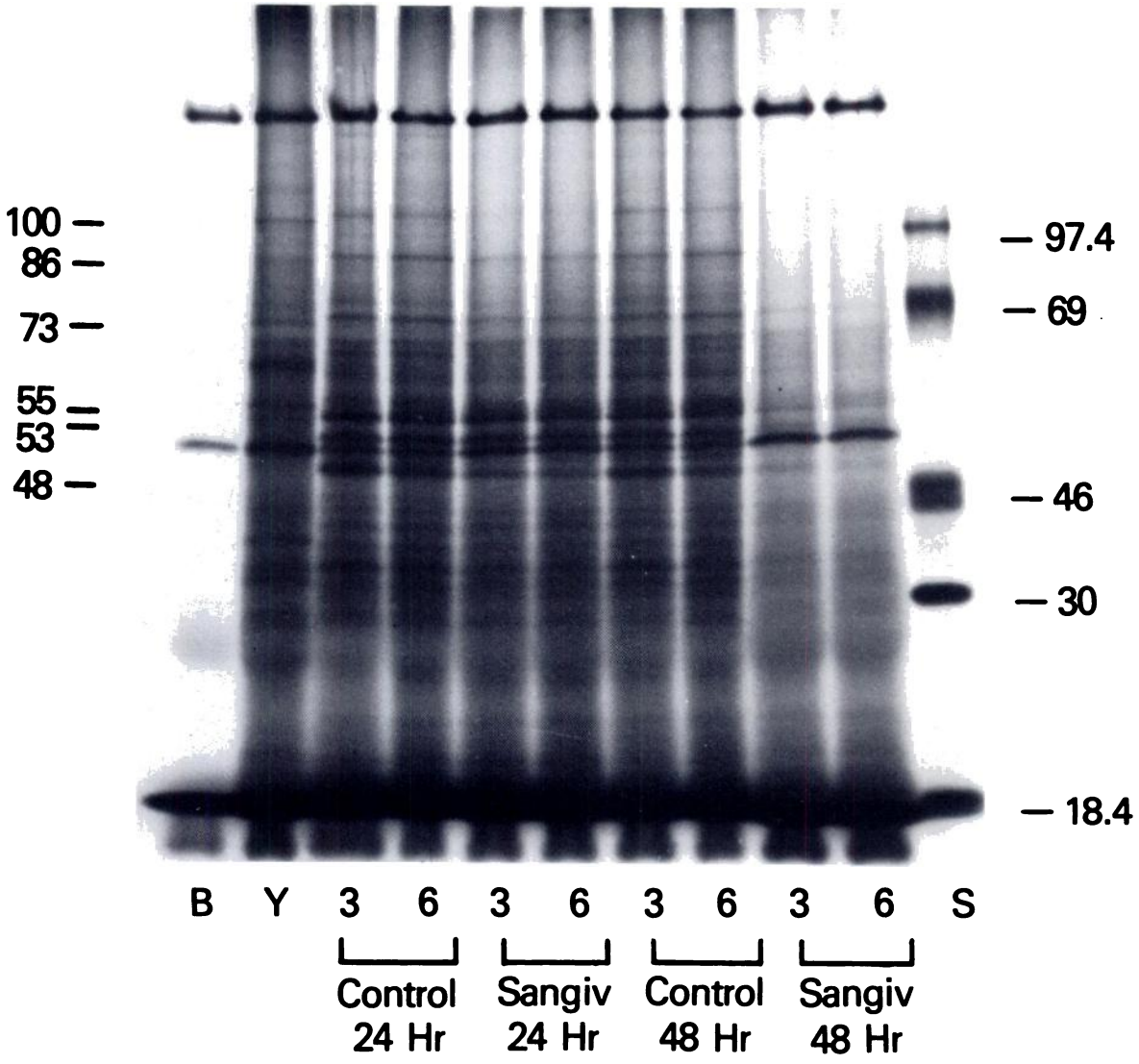

FIG. 2. *Polyacrylamide gel electrophoretogram of [^{35}S]methionine-labeled proteins translated in vitro*

Cells were treated as described in Fig. 1, and isolated mRNA was translated *in vitro* using a reticulocyte lysate system as described under Experimental Procedures. An autoradiogram of the translated products is shown. *B*, blank assay without exogenous mRNA; *Y*, yeast mRNA standard. The slots designated *3* and *6* represent the amount (micrograms) of mRNA added to the assay. *Sangiv*, sangivamycin.

DISCUSSION

Previous studies of the mechanism of action of sangivamycin indicated that the antibiotic produced a markedly time-dependent cytocidal effect (1, 2) which correlated closely with the incorporation of drug into mRNA (2, 5). Of particular note was the minimal effect of the drug on post-transcriptional processes such as methylation (3, 4) and polyadenylylation (3) and its weak to moderate inhibitory effect on transcription (1–4). The present investigation confirms the inhibitory action of sangivamycin on RNA synthesis, and, more importantly, shows that treatment of HT-29 cells by this drug under defined conditions of cell lethality results in a lower translational activity *in vitro* of drug-modified mRNA. Since the percentage of poly(A)RNA from control and drug-treated cells was comparable, these results suggest that inhibition of translation directed by total RNA from sangivamycin-treated cells is not a direct result of a lesser mRNA content.

Whether the reduced translational activity produced by sangivamycin treatment is the result of miscoding or a decreased affinity of the mRNA for ribosomal binding sites is not known. Although no new proteins were apparent after either treatment interval, the 48-hr treatment period did result in the disappearance of at least two major proteins of 86,000 and 100,000 mol wt (Fig. 2). These results suggest some selectivity to the action of the drug since the reduced translation of these proteins was also apparent after the shorter treatment interval.

It is still unclear whether reduced mRNA activity *in vitro* is responsible for cell lethality. Since mRNA activity was reduced by only 30% and 40–50% after 24 and 48 hr of treatment, respectively, in comparison to 83% and 97% reductions in cell viability (2), this effect may be only partially responsible for cell lethality or a phenomenon secondary to cell death. On the other hand, it may not be necessarily accurate to assume an arithmetic relationship between these parameters if a crucial growth-associated peptide is affected. A third possibility is that the action of sangivamycin on translation *in situ* may be quite different both quantitatively and qualitatively from that effected *in vitro*. Further studies with a specific complementary DNA probe or peptide product may help to clarify some of these questions.

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