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COMPARISON BETWEEN THE INHIBITORY ACTIVITIES OF SANGIVAMYCIN AND THIOSANGIVAMYCIN ON NUCLEAR RIBONUCLEIC ACID SYNTHESIS IN L1210 CELLS *IN VITRO*

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Abstract—The molecular effects of the pyrrolopyrimidine analogs, sangivamycin and thiosangivamycin, on RNA and DNA synthesis were examined in L1210 cells *in vitro*. Pretreatment of cells for 30 min with either sangivamycin or thiosangivamycin resulted in a median inhibitory dose of 1×10^{-5} M and 2×10^{-5} M, respectively, for RNA synthesis. Sangivamycin did not inhibit DNA synthesis at comparable concentrations, while thiosangivamycin inhibited DNA synthesis by 20–35 per cent. Extending preincubation times with either drug to 1 hr resulted in decreased inhibition of RNA synthesis. Sangivamycin and thiosangivamycin also inhibited the transcription of nuclear RNA with ^{32}P as precursor. Incorporation of ^{32}P into AMP was reduced by both drugs to a much greater extent that the labeling of GMP, CMP, or UMP of alkaline hydrolysates of total nuclear RNA. Thiosangivamycin also significantly reduced methylation in nuclear rRNA. Neither drug affected the concentration or specific radioactivity of *S*-adenosyl-L-methionine or UTP. Sangivamycin and thiosangivamycin inhibited the syntheses of nuclear rRNA, non-poly(A) heterogeneous RNA and poly(A) heterogeneous RNA to equal extents, but the potency of sangivamycin was approximately 10-fold less than that of thiosangivamycin. These results suggest that sangivamycin and thiosangivamycin act as adenosine analogs to inhibit the transcription of all forms of nuclear RNA in L1210 cells.

Several adenosine analogs have proved to be effective anticancer drugs against murine leukemias when administered with the adenosine deaminase inhibitor, 2′-deoxycoformycin [1–3]. However, it would be advantageous to utilize analogs whose activity would not be compromised by deamination, and thus eliminate the inconvenience of a two-drug regimen. Two such adenosine analogs are the pyrrolopyrimidines, sangivamycin and thiosangivamycin (Fig. 1). Sangivamycin is readily phosphorylated by adenosine kinase *in vitro* [4] and is not deaminated [5]. This antibiotic is also incorporated into RNA and DNA of several normal tissues in mice [6] and inhibits *de novo* purine synthesis in L1210 cells in culture [7]. Moreover, *in vitro* studies with sangivamycin 5′-triphosphate have demonstrated that this metabolite can competitively inhibit amino acid activation, and hence, charging of tRNA [8], as well as substitute for ATP and inhibit the synthesis of RNA catalyzed by *Escherichia coli* RNA polymerase [9].

Since sangivamycin and thiosangivamycin are effective antitumor agents against several murine tumors (J. Plowman, personal communication), and the former drug is of clinical interest [10], studies were initiated to examine their mechanisms of action on nucleic acid synthesis.

MATERIALS AND METHODS

Materials. Sangivamycin (NSC 65346) and thiosangivamycin (NSC 105827) were provided by Dr. John Douros, Natural Products Branch, National Cancer Institute. [5,6-^{3}H]Uridine (41.3 Ci/mmole), [U-^{14}C]uridine (464 mCi/mmole), [5-*methyl*]-thymidine (20 Ci/mmole), [^{3}H-*methyl*]-L-methionine (80 Ci/mmole), [2,8-^{3}H]adenosine (35.2 Ci/mmole) and ^{32}P as orthophosphoric acid in HCl-free water were obtained from the New England Nuclear Corp., Boston, MA.

Animals. L1210 cells were maintained as described previously [11].

Incubations. Incubations were carried out at 37° in a shaking water bath at 100 rev/min and consisted of: 5 ml RPMI 1630 medium, either 10 μCi [^{3}H]uridine (200 mCi/mmole) or 10 μCi [^{3}H]-thymidine (200 mCi/mmole), drug and 1×10^{7} cells. When nRNA* was extracted, each assay was increased 5-fold with respect to the number of cells, volume of medium and amount of isotope. In the latter instance, 50 μCi [^{3}H]adenosine (35.2 Ci/mmole), 1 mCi ^{32}P (in phosphate-free RPMI 1630 supplemented with 20 mM Hepes, pH 7.4) or 5 μCi

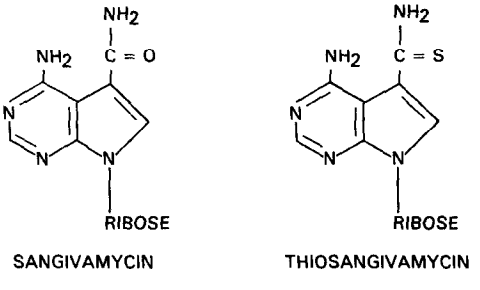

Fig. 1. Structures of sangivamycin and thiosangivamycin.

* Abbreviations used are: rRNA, nuclear RNA; hnRNA, heterogeneous RNA; poly(A), polyadenylic acid; ID_{50}, 50 per cent inhibitory dose; and Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

[^{14}C]uridine (464 mCi/mmole) plus 50 μCi [^{3}H-*methyl*]methionine (80 Ci/mmole) in Dulbecco's medium supplemented with 0.25% glucose were used for labeling nRNA, depending on the experimental protocol.

Measurement of total RNA and DNA syntheses. Incorporated [^{3}H]uridine and [^{3}H]thymidine into total RNA and DNA, respectively, was measured by precipitation and filtration of trichloroacetic acid-insoluble material on glass fiber filter discs [12].

RNA extraction. After incubation, cells were centrifuged at 400 *g* for 15 min at 4° and washed once with RPMI 1630 medium. Nuclei were prepared by the method of Daskal *et al.* [13] using Triton X-100. Total nRNA, as well as nuclear rRNA, non-poly(A)hnRNA and poly(A)hnRNA were extracted by the sodium dodecyl sulfate–phenol procedures described previously [11]. Poly(A)hnRNA was isolated by poly(U)Sepharose chromatography [14]. Poly(A) was isolated from poly(A)hnRNA by digestion with RNase A and RNase T_1 [11, 15].

High voltage electrophoresis. Total nuclear RNA was hydrolyzed with 0.3 N KOH overnight at 37°. Digests were neutralized with perchloric acid and lyophilized. Aliquots of the reconstituted RNA hydrolysate were spotted on flexible cellulose-coated thin-layer sheets (Eastman Kodak, Rochester, NY) and electrophoresed in an MRA Corp. (Clearwater, FL) high voltage electrophoresis apparatus with 5% (v/v) acetic acid (adjusted to pH 3.5 with NaOH) as the buffer and orange G and the tracking dye. Nucleotides were located by u.v. absorption using appropriate standards run in parallel. Thin-layer sheets were cut into 0.5 cm strips and counted in 10 ml Aquasol.

DEAE Sephadex-urea chromatography. Mononucleotide, dinucleotide and oligonucleotide fractions of alkaline hydrolysates of total nRNA were separated as described previously [16].

S-Adenosyl-L-methionine and UTP analyses. The concentrations and specific radioactivities of UTP and *S*-adenosyl-L-methionine were measured in neutralized perchloric acid extracts of L1210 cells using assay conditions identical to those for double labeling with [^{3}H-*methyl*]methionine and [^{14}C]uridine described above. *S*-Adenosyl-L-methionine was assayed by SP Sephadex chromatography as described previously [17], and UTP was assayed by Dowex chromatography [18].

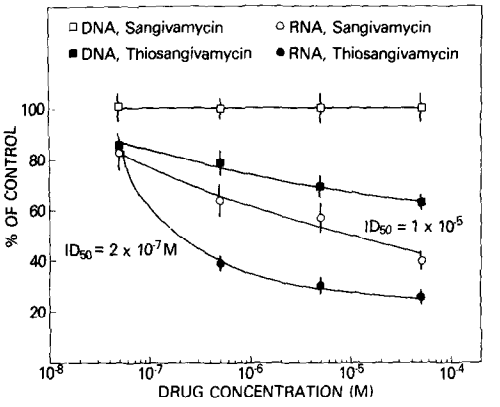

Fig. 2. Dose-response of sangivamycin and thiosangivamycin on RNA and DNA syntheses. L1210 cells (10^{7} cells/flask) were preincubated for 30 min with either sangivamycin or thiosangivamycin and then labeled for 30 min with either [^{3}H]uridine or [^{3}H]thymidine to measure RNA and DNA synthesis respectively. Each value is the mean ± S.E. of four determinations.

RESULTS

The initial experiments were designed to determine the specificity of action and ID_{50} of sangivamycin and thiosangivamycin on RNA and DNA syntheses (Fig. 2). Sangivamycin inhibited RNA synthesis by 50 per cent at 1 × 10^{-5} M, while not affecting DNA synthesis. The median inhibitory dose of the thio-

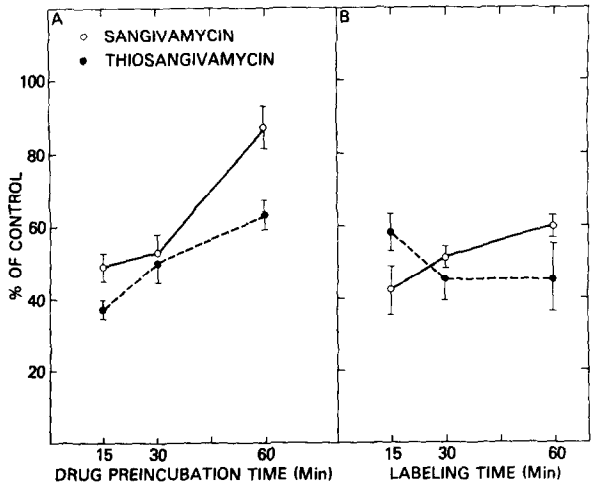

Fig. 3 Effects of drug preincubation time and labeling time on the inhibition of RNA synthesis by sangivamycin and thiosangivamycin. L1210 cells (10^{7} cells/flask) were either preincubated with drug for (A) 15, 30 or 60 min and then labeled with [^{3}H]uridine for 30 min or (B) preincubated with drug for 30 min and then labeled with [^{3}H]uridine for either 15, 30 or 60 min. Results are expressed as per cent of control. Each value represents the mean ± S.E. of four determinations.

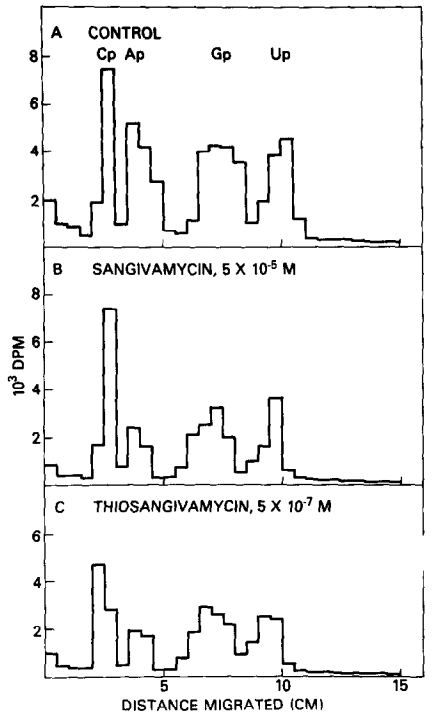

Fig. 4. High voltage electrophoresis of alkaline hydrolysates of total nRNA labeled with ^{32}P. L1210 cells (5×10^7 cells/flask) were preincubated for 30 min with either 5×10^{-4} M sangivamycin or 5×10^{-7} M thiosangivamycin and then labeled for 30 min with 1 mCi of ^{32}P.

sangivamycin was 50-fold lower than for sangivamycin; however, DNA synthesis also proved sensitive to this drug, but an ID_{50} was not achieved at concentrations up to 5×10^{-5} M.

To assess the influence of drug preincubation and pulse-labeling times on the inhibition of RNA synthesis by the two pyrrolopyrimidines, temporal studies were carried out (Fig. 3). A consistent reduction in inhibitory potency was noted for both inhibitors during prolonged preincubation (Fig. 3A). This effect was particularly notable with sangivamycin; only 15 per cent inhibition was observed after 60 min of preincubation vs 50 per cent after 30 min. With thiosangivamycin, inhibition was diminished from 50 per cent at 30 min to 35 per cent at 60 min. Varying the time of pulse-labeling, with 30 min of drug preincubation, did not produce significant differences in the degree of inhibition by the two drugs. Thus, these data suggest that sangivamycin is less metabolically stable than thiosangivamycin prior to its utilization for inhibiting transcription.

In order to test whether or not sangivamycin and thiosangivamycin acted as adenosine analogs to specifically impair the utilization of ATP for transcription, nRNa was labeled with ^{32}P. Alkaline hydrolysates of total nRNA were subjected to high voltage electrophoresis, and the incorporated ^{32}P in each of the four ribonucleotides was measured (Fig. 4, Table 1). Both inhibitors reduced the incorporation of ^{32}P into all four nucleotides, but labeling of AMP was significantly lower vs GMP, CMP and UMP (Table 1). Although a new nucleotide peak resulting from drug incorporation was not apparent (Fig. 4), the sensitivity of the experiment was such that substitution of less than 1.0 per cent could not be detected, and thus, incorporation of low levels of the drugs into RNA is not completely ruled out. In addition, it is possible that electrophoretic migration of the 3′-phosphate of sangivamycin or thiosangivamycin could coincide with one of the four natural ribonucleotides and mask analysis of the drug. However, the results of Swart and Hodge [19], showing a different electrophoretic migration for the closely related pyrrolopyrrimidine, toyocamycin 3′-phosphate, from the four ribonucleotides, would appear to make the latter possibility unlikely.

Since transcription appeared to be reduced significantly by sangivamycin and thiosangivamycin, it was of interest to examine if the post-transcriptional process of methylation was equally impaired by these

Table 1. Distribution of ^{32}P in alkaline hydrolysates of total nRNA from L1210 cells treated with sangivamycin and thiosangivamycin*

	Control		Sangivamycin (5×10^{-5} M)		Thiosangivamycin (5×10^{-7} M)	
Hydrolysate	dis./min	% Distribution of radioactivity	dis./min	% Distribution of radioactivity	dis./min	% Distribution of radioactivity
Cp	11,090 ± 1,020 (100)	23.5 ± 2.2	7,120 ± 950† (64)	27.8 ± 0.7	6,500 ± 610† (59)	27.7 ± 0.9
Ap	10,610 ± 1,350 (100)	22.7 ± 0.7	3,630 ± 470‡ (34)	14.6 ± 0.7‡	3,630 ± 360‡ (34)	15.7 ± 1.5‡
Gp	14,380 ± 1,780 (100)	30.3 ± 1.4	8,500 ± 1,050† (59)	34.2 ± 0.7	7,620 ± 780† (53)	32.0 ± 1.7
Up	11,300 ± 1,150 (100)	23.9 ± 0.3	5,950 ± 780† (53)	23.4 ± 0.4	5,940 ± 640† (53)	24.6 ± 1.1
Total	47,380 ± 5,270 (100)		25,200 ± 3,240† (53)		23,690 ± 4,530† (50)	
$\frac{A+U}{G+C}$	0.86 ± 0.03		0.61 ± 0.02‡		0.68 ± 0.03‡	

* Values represent the means ± S.E. of three determinations. Numbers in parentheses indicate percentages of control dis./min.

† Statistically significant difference ($P < 0.05$) vs control.

‡ Statistically significant difference ($P < 0.01$) vs control.

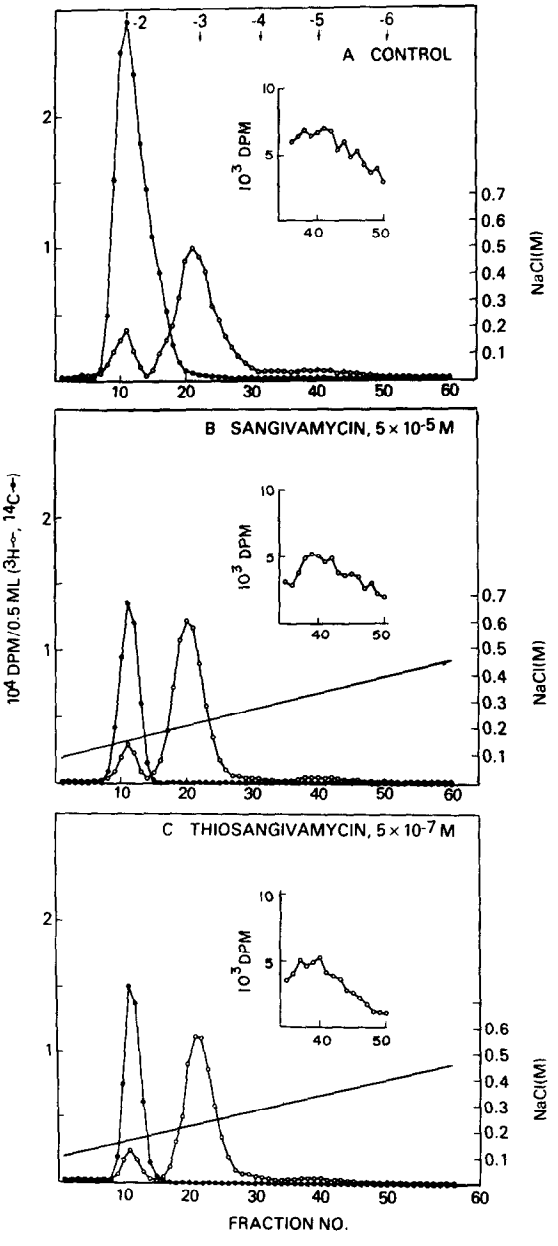

Fig. 5. DEAE Sephadex-urea chromatography of alkaline hydrolysates of nRNA. L1210 cells (5×10^7 cells/flask) were preincubated for 30 min with either 5×10^{-5} M sangivamycin or 5×10^{-7} M thiosangivamycin and then labeled for 30 min with [^{3}H-*methyl*]L-methionine and [^{14}C]uridine. The "inset" represents an expansion in scale of the −5 to −6 region of the chromatographic profile.

drugs. Alkaline hydrolysates of total nRNA were chromatographed on DEAE Sephadex, and mono-, di- and oligonucleotide fractions were isolated by their net charges of -2, -3 and -5 to -6 respectively (Fig. 5). It was found that there was a small reduction in methylation of total nRNA by only thiosangivamycin despite the extensive inhibition of labeling with [^{14}C]uridine by both drugs.

Further analysis of nRNA was carried out by its fractionation into rRNA, non-poly(A)hnRNA and poly(A)hnRNA (Fig. 6). Double-labeling experiments with [^{3}H-*methyl*]methionine and [^{14}C]uridine indicated a dose-dependent reduction in [^{14}C]uridine incorporation into all species of nRNA by sangivamycin and thiosangivamycin. In addition, thiosangivamycin specifically reduced the methylation of rRNA at 5×10^{-7} M but did not significantly affect the methylation of other fractions of nRNA. In all instances, inhibition of methylation by thiosangivamycin was considerably less that inhibition of [^{14}C]uridine incorporation.

In order to rule out artifactual changes in RNA synthesis and methylation via changes in the pool size and specific radioactivity of UTP and *S*-adenosyl-L-methionine, respectively, measurements of these metabolities in L1210 cells were performed (Table 2). No significant alterations in either the concentration or specific radioactivity of *S*-adenosyl-L-methionine or UTP were found as a result of inhibitory concentrations of sangivamycin and thiosangivamycin.

Analyses of nuclear rRNA, non-poly(A)hnRNA, and poly(A)hnRNA, as well as poly(A) labeled with [^{3}H]adenosine in the presence of varying concentrations of sangivamycin and thiosangivamycin, are presented in Fig. 7. The extent of inhibition produced by these drugs was similar to that with [^{14}C]uridine as a precursor (Fig. 6). No differential effects occurred except for a slightly lesser degree of sensitivity of poly(A)hnRNA and poly(A) to thiosangivamycin vs rRNA and non-poly(A)hnRNA (Fig. 7B).

DISCUSSION

The present study documents that the antibiotic, sangivamycin, and its synthetic analog, thiosangivamycin, are potent inhibitors of nRNA synthesis. They appear to be fairly specific in action since DNA synthesis was only slightly affected or unaffected by concentrations of drug which markedly impaired RNA synthesis. The present experiments also suggest that these pyrrolopyrimidines act as adenosine analogs since they reduced the labeling and incorporation of adenosine in nRNA (Table 1). The latter effect appears to be a result of competition with adenosine for utilization by adenosine kinase [4], as well as impairment of transcription via the 5′-triphosphate metabolite [9].

Three differences between sangivamycin and thiosangivamycin were noted. First, sangivamycin was less stable than the thio analog with long preincubation times (Fig. 3) which may reflect differences in catabolism. Second, thiosangvamycin reduced the methylation of rRNA, whereas only slight inhibition was observed with sangivamycin. Third, thiosangivamycin, although a more potent inhibitor of RNA synthesis than of DNA synthesis, nevertheless impaired DNA synthesis throughout the concentration range studied. It would appear from these observations that thiosangivamycin is less specific in its mode of action than sangivamycin. In addition, the greater potency of the thio analog is probably associated with its greater metabolic stability.

Although sangivamycin and thiosangivamycin inhibited transcription, it is not known if this biochemical effect is responsible for their antitumor

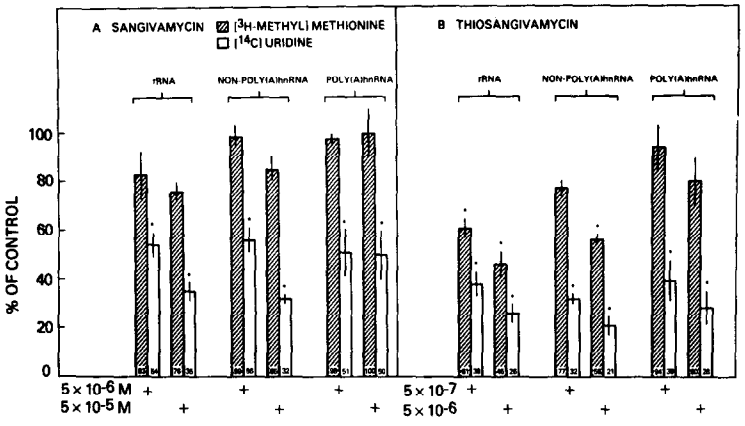

Fig. 6. Effects of sangivamycin and thiosangivamycin on the methylation and synthesis of nRNA. L1210 cells (5×10^7 cells/flask) were preincubated for 30 min with either (A) sangivamycin or (B) thiosangivamycin and then labeled for 30 min with [^{3}H-*methyl*]-L-methionine and [^{14}C]uridine. Nuclear RNA was fractionated into rRNA, non-poly(A)hnRNA and poly(A)hnRNA as described under Materials and Methods. Results are expressed as per cent of control. Each value represents the mean ± S.E. of three determinations. Asterisks (*) indicate statistically significant differences ($P < 0.05$) vs controls.

Table 2. Effects of sangivamycin and thiosangivamycin on the concentration and specific radioactivity of *S*-adenosyl-L-methionine and UTP*

Treatment	*S*-adenosyl-L-methionine		UTP	
	nmoles/5 × 10^7 cells	10^3 dis/min/nmole	nmoles/5 × 10^7 cells	dis./min/nmole
Control	11.9 ± 0.2 (100)	362.0 ± 33.1 (100)	99.8 ± 3.9 (100)	5780 ± 280 (100)
Sangivamycin (5×10^{-5} M)	11.8 ± 0.8 (99)	391.2 ± 42.3 (108)	106.5 ± 7.2 (107)	5130 ± 120 (89)
Thiosangivamycin (5×10^{-7} M)	11.8 ± 0.5 (99)	370.6 ± 46.4 (102)	106.5 ± 5.3 (107)	6100 ± 230 (106)

* Values for control and drug-treated cells represent the means ± S.E. of six determinations. Numbers in parentheses represent percentages vs control = 100 per cent.

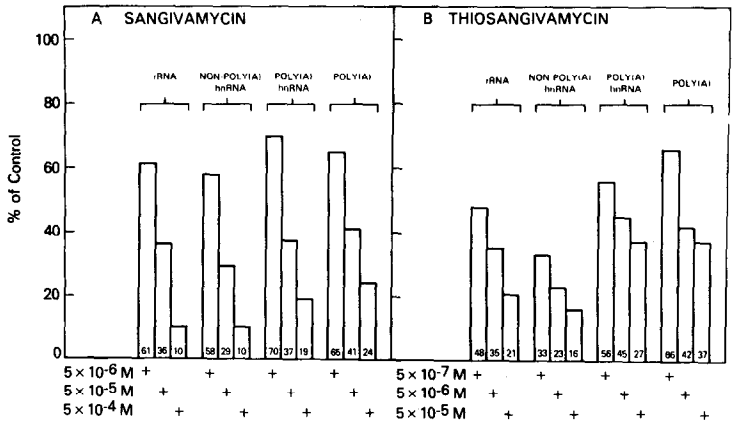

Fig. 7. Effects of sangivamycin and thiosangivamycin on the incorporation of [^{3}H]adenosine into nRNA. L1210 cells (5×10^7 cells/flask) were preincubated for 30 min with (A) sangivamycin or (B) thiosangivamycin, and then labeled for 30 min with [^{3}H]adenosine. Results are expressed as per cent of control. Each value represents the mean of three determinations.

effects. Inhibition of *de novo* purine synthesis by $0.1–1 \times 10^{-6}$ M sangivamycin [7], as well as incorporation of sangivamycin into RNA [5, 9], have been reported. Although we did not find evidence for the latter activity, we cannot completely rule out low levels (less than 1 per cent) of incorporation.

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