MOLECULAR PHARMACOLOGY, 27:349–355

Comparison of the Cellular and RNA-Dependent Effects of Sangivamycin and Toyocamycin in Human Colon Carcinoma Cells

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Received August 30, 1984; Accepted December 31, 1984

SUMMARY

The effects of the pyrrolopyrimidine antibiotics sangivamycin and toyocamycin on the synthesis of RNA and protein, ribosomal RNA processing, and cell viability were examined in colon carcinoma cell line HT-29. Exposure for 24 hr to toyocamycin caused an exponential type of cell lethality resulting in a 4-log reduction of cell viability, while sangivamycin produced a gradual and self-limiting type of cell lethality resulting in a 1-log reduction of cell viability. Toyocamycin, at a concentration of 1 μM produced total cessation of precursor rRNA processing, while 10 μM sangivamycin produced little or no effect on processing. On the contrary, sangivamycin caused a significant decrease in protein synthesis after 6 hr, while toyocamycin had less effect. The inhibition of protein synthesis by sangivamycin results from an inhibition of the formation of complexes essential to the initiation of protein synthesis. The results suggest that the mechanisms of action of these closely related agents are quite distinct. The marked loss of cell viability caused by toyocamycin correlates with its effect on rRNA processing, while the slow inhibition of protein synthesis appears to be secondary to the loss of ribosome synthesis. On the other hand, the lesser cytotoxicity produced by sangivamycin results from a more direct effect on protein synthesis. Importantly, cells are much less capable of resuming normal proliferative activity after 24 hr of impaired rRNA processing than after a similar interval of reduced protein synthesis.

INTRODUCTION

The pyrrolopyrimidine nucleoside antibiotics sangivamycin, toyocamycin, and tubercidin are cytotoxic to mammalian cells in culture and growth inhibitory to a wide variety of bacteria, fungi, parasites, DNA viruses, and double- and single-stranded RNA viruses (1). There is renewed clinical interest in these compounds, which were originally inadequately tested as cancer chemotherapeutic agents. In particular, sangivamycin is undergoing new clinical trials (2) since the discovery that its lethal effect is highly dependent on the duration of drug exposure (3).

As analogs of adenosine, these compounds compete with adenine nucleotides in such cellular processes as nucleic acid biosynthesis (1, 4, 5), transmethylation (6), and amino acid activation (7). Toyocamycin inhibits the conversion of the high molecular weight precursor of rRNA to its mature forms (8). In this report, we compare the effects of sangivamycin and toyocamycin (Fig. 1) on cell viability, RNA synthesis and processing, and protein synthesis, and we find that their mechanisms of action are dissimilar.

MATERIALS AND METHODS

Materials. [5-^{3}H]Urd (26.2 Ci/mmol), [U-^{14}C]Urd (506 mCi/mmol), L-[3,4,5-^{3}H]leucine (146 Ci/mmol), and L-[*methyl*-^{3}H]methionine (10 Ci/mmol) were purchased from New England Nuclear Corporation (Boston, MA). Sangivamycin and toyocamycin were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute (Bethesda, MD), and actinomycin D was purchased from Sigma Chemical Corporation (St. Louis, MO).

Cell culture. The growth properties of human colon carcinoma cell line HT-29 have been described previously (9). Cells were grown in monolayers at 37° and 5% CO_2 in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum, gentamicin, 50 μg/ml, and 40 mM Hepes,[1] pH 7.4. Cell inocula consisted of 10^5 cells/10 ml or 10^6 cells/100 ml in 25-cm^2 or 150-cm^2 plastic flasks, respectively. Cells were allowed 3 days to enter logarithmic growth before drugs were added. Cells were harvested by trypsinization as described previously (3). Cells processed for RNA extraction were prelabeled for 48 hr with 0.025 μCi/ml [^{14}C]Urd (506 mCi/nmol), after which the cells were grown in fresh media for 24 hr and then labeled either for 2 hr with 1.0 μCi/ml [^{3}H]Urd (26.2 Ci/mmol) or for 24 hr with 0.2 μCi/ml [^{3}H]Urd (500 mCi/mmol).

Cell viability. The soft agar clonogenic assay was performed as described previously (3), except that the cloning medium contained 40 mM Hepes, pH 7.4. Cell viability is expressed as the number of surviving colonies from drug-treated cells divided by the number of colonies from

[1] The abbreviations used are: Hepes; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PBS, phosphate-buffered saline, (6.3 mM Na_2HPO_4, 0.8 mM NaH_2PO_4, 0.154 M NaCl, pH 7.4); SDS, sodium lauryl sulfate.

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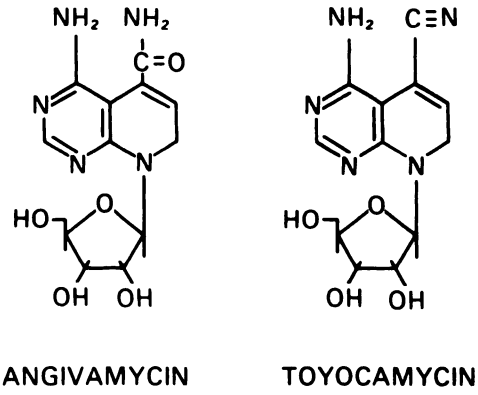

FIG. 1. *The structure of sangivamycin and toyocamycin*

control cells × 100. Cloning efficiency ranged from 30 to 40%. Cells were judged to be intact and suitable for RNA extraction after drug exposure by their ability to exclude trypan blue dye.

RNA and protein synthesis. The rates of RNA and protein synthesis were determined by the incorporation of [^{14}C]Urd and [^{3}H]leucine, respectively. Cells were labeled during the last hour of treatment with 0.1 μCi/ml of [^{14}C]Urd (506 mCi/mol) and 0.5 μCi/ml of [^{3}H]leucine (146 Ci/mmol, 1.3 mCi/mmol after dilution by the leucine in the medium), washed with Hanks' balanced salt solution, trypsinized, precipitated with cold 10% trichloroacetic acid/2% pyrophosphate and collected on glass fiber discs (GF/B, Whatman Laboratory Products, Clifton, NJ). Neither sangivamycin nor toyocamycin affected the levels of the four ribonucleoside triphosphates as measured by anion-exchange chromatography as described previously (10). Also, the compounds did not significantly affect the specific radioactivity of the cellular UTP pool.

Extraction of whole cell RNA. Cell monolayers were washed once, scraped into ice-cold PBS, and collected by centrifugation. The cells were resuspended in 1.8 ml of extraction buffer (0.02 M sodium acetate, 0.14 M NaCl, 10 μg/ml polyvinyl sulfate, pH 5.0) and adjusted to 0.2% in SDS (200 μl of 2% SDS) while shaking on a Vortex mixer at room temperature. After 1 min, 2 ml of phenol (water saturated and containing 0.1% 8-hydroxyquinoline) was added, mixed 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, and finally the aqueous phase was reextracted with fresh phenol for a third time. Three volumes of 95% ethanol/2% potassium acetate was 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 × 0.5 cm cylindrical gels containing from 1.9 to 2.1% polyacrylamide and 0.6% agarose in buffer (0.04 M Tris, 0.02 M sodium acetate, 0.003 M EDTA, 10% glycerol, pH 7.6) (11). Running buffer consisted of the above buffer plus 0.3% SDS. Loading buffer consisted of running buffer containing 20% glycerol. Gels were run at 6 mA/gel for 3 hr. Gel slices (2.2 mm) were dissolved in concentrated perchloric acid and neutralized with 8 N NaOH, and radioactivity was determined by liquid scintillation counting. *Note:* gels containing greater than 2.1% polyacrylamide will not dissolve in perchloric acid.

Initiation complex formation assay. Cells grown in 150-cm^2 flasks were prelabeled with 0.02 μCi/ml of [^{3}H]Urd (100 mCi/mmol) for 2 days followed by 1 day in nonradioactive medium. After incubation with drug for 5.5 hr, the cells were washed with methionine-free medium (all methionine-free media contained the drug under study), trypsinized, washed again with methionine-free medium, and resuspended in 0.5 ml of methionine-free medium containing 0.1 mM sparsomycin to inhibit elongation. Inhibiting elongation eliminates contamination by proteins containing [^{35}S]methionine and the release and deaggregation of the 80 S initiation complex (12). Cells were preincubated for 20 min and incubated with 200 μCi of [^{35}S]methionine for 10 min. Incubations were halted by pelleting the cells with a 3-sec centrifugation in an Eppendorf centrifuge and washing three times with ice-cold PBS. The pellet was homogenized in 1.0 ml of 20 mM Hepes, pH 7.5, 75 mM KCl, 2.5 mM magnesium acetate, 0.5% Nonidet P-40 in a Dounce homogenizer, and the homogenate was centrifuged at 10,000 × *g* for 10 min. The supernatant was layered on a 10 to 30% glycerol gradient in the above buffer without Nonidet P-40 and centrifuged at 39,000 rpm at 4° for 3.5 hr in an SW 41 rotor (13). Fractions were collected from the bottom of the tube and collected on Whatman GF/B glass fiber dishes by precipitation with 10% trichloroacetic acid.

RESULTS

Cell viability. The viability of cells exposed for 2 or 24 hr to various concentrations of either sangivamycin or toyocamycin was determined by a soft agar clonogenic assay (Fig. 2). Exposure of cells for 2 hr to either compound was not cytotoxic. However, the effect on viability of a 24-hr exposure to the two agents was dramatically different. Sangivamycin caused a gradual loss of viability to 7% survival over a wide concentration range. In contrast, exposure to toyocamycin resulted in a 4-log reduction of viability over a narrow concentration range. The effect of exposure to 1 μM toyocamycin for various times is also presented in Fig. 2; 2-hr exposure had no effect on viability, while a 6-hr or 12-hr treatment reduced viability to 6 or 0.8% of control, respectively. Incubation with 1 μM toyocamycin for either 18 or 24 hr resulted in a loss of cell viability which exceeded the detection limit of the assay (greater than 4 logs).

RNA and protein synthesis. The synthesis of RNA and protein was measured by the incorporation of [^{14}C]Urd and [^{3}H]leucine, respectively (Fig. 3). The effects of sangivamycin and toyocamycin on both RNA and protein synthesis were dissimilar. Protein synthesis was inhibited more after treatment with sangivamycin than after exposure to toyocamycin (Fig. 3A), while toyocamycin was a more potent inhibitor of RNA synthesis

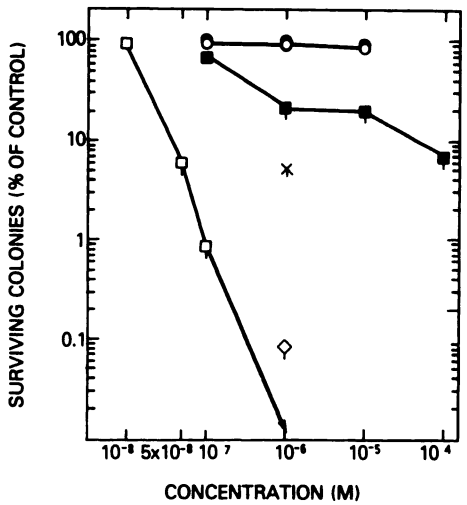

FIG. 2. *Viability of HT-29 cells after exposure to sangivamycin or toyocamycin*

Cells were exposed for 2 hr to sangivamycin (●) or toyocamycin (○), for 6 (×) or 12 hr (◇) to 1 μM toyocamycin, or 24 hr to sangivamycin (■) or toyocamycin (□) and the viability was determined by soft agar colony formation as described in Materials and Methods. Each value is the mean of four determinations.

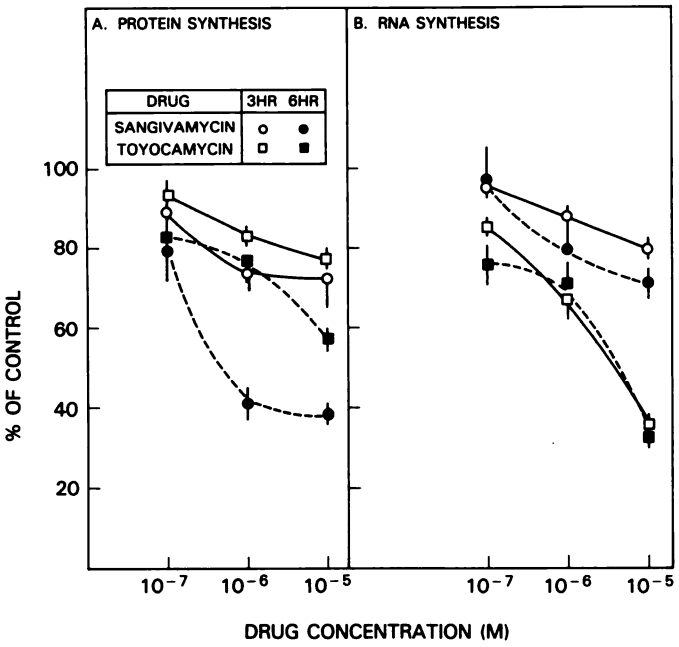

FIG. 3. *Incorporation of [^{3}H]leucine into protein and [^{14}C]Urd into RNA after exposure to sangivamycin or toyocamycin*

Cells were exposed to sangivamycin or toyocamycin during 3 or 6 hr. 0.5 μCi/ml of [^{3}H]Leu (146 Ci/mmol) and 0.1 μCi/ml of [^{14}C]Urd (506 mCi/mmol) were added for the final hour of exposure. Protein and RNA were analyzed as described in Materials and Methods. Each value is the mean of six determinations.

than sangivamycin (Fig. 3B). The time period between 3 and 6 hr appeared critical for the expression of the inhibitory effects of sangivamycin on protein synthesis. In contrast, the effect of toyocamycin on RNA synthesis was fully evident at 3 hr.

The effects of sangivamycin and toyocamycin on RNA and protein synthesis were compared to those of 0.05 μg/ml actinomycin D, a concentration that preferentially inhibits rRNA synthesis. RNA synthesis was 50 and 37% of control after 3-hr and 6-hr incubations with actinomycin D, respectively, while protein synthesis was 99 and 77% at these times (results not shown). Therefore, the drastic inhibition of protein synthesis by sangivamycin is not explained by a lack of newly synthesized ribosomes, but appears to result from a more direct effect. In contrast, the effects of toyocamycin on protein synthesis resemble those resulting from a lack of newly synthesized ribosomes.

Effects on rRNA processing. Total RNA from cells labeled with [^{3}H]Urd and incubated with various concentrations of either sangivamycin or toyocamycin was analyzed by polyacrylamide-agarose gel electrophoresis in order to observe their effects on the processing of rRNA (Figs. 4–7). Results are presented for 1 μM toyocamycin and 10 μM sangivamycin. The effect of 1 μM sangivamycin (data not shown) was similar to that of 10 μM sangivamycin. Prelabeling of cells with [^{14}C]Urd allowed unambiguous identification of 28 S and 18 S rRNA and simplified the identification of rRNA precursors. After 2 hr of labeling with 1.0 μCi/ml of [^{3}H]Urd, untreated cells contained significant amounts of radioactivity in rRNA precursors (45 S and 32 S), mature rRNA (28 S and 18 S), as well as in heterogeneous RNA (60 S–90 S) (Fig. 4A). After 2 hr of labeling concurrent with sangivamycin treatment, there was a slight change in the profile of rRNA which indicated a deficiency of 28 S rRNA relative to 18 S rRNA (Fig. 4B). This result could be consistent with a reduced ability to process 32 S rRNA to mature 28 S rRNA. In contrast, 2 hr of labeling concurrent with toyocamycin treatment resulted in a total lack of synthesis of mature rRNA, reduced 32 S rRNA, and slightly increased 45 S rRNA (Fig. 4C). These results are consistent with a reduced ability to process the 45 S rRNA precursor to mature rRNA. This effect also was previously found in HeLa, Novikoff hepatoma, and L5178Y cells treated with toyocamycin (8, 14, 15). Labeling during the final 2 hr of a 24-hr incubation with either sangivamycin or toyocamycin showed a drastic inhibition of transcription (Fig. 5). To observe clearly the buildup of unprocessed precursor rRNA, cells were labeled for 24 hr with 0.2 μCi/ml of [^{3}H]Urd (500 mCi/mmol) concurrent with drug treatment. In untreated cells, the amount of labeled mature rRNA continuously accumulated, while the amount of labeled RNA precursors reached and remained at a much lower steady state level (Fig. 6A). Therefore, significant radioactivity was only observed in mature 28 S and 18 S rRNA. After incubation with sangivamycin, most of the radioactivity was still in mature 28 S and 18 S rRNA, while 45 S and 32 S rRNA precursors contained little radioactivity (Fig. 6B). The deficiency of 28 S rRNA relative to 18 S rRNA observed during short term labeling was no longer present. However, after incubation with toyocamycin, there was a large buildup of 45 S rRNA, while no label was observed in 32 S, 28 S, or 18 S rRNA (Fig. 6C). Therefore, 1 μM toyocamycin completely inhibited the processing of 45 S precursor, while 10 μM sangivamycin had minimal, if any, lasting effect on the processing of rRNA.

Methylation of rRNA. Hypomethylation of rRNA also can result in processing defects (16). To observe the methylation state of rRNA, cells were labeled with 4.0 μCi/ml of [*methyl*-^{3}H]methionine (10 Ci/mmol) in methionine-free medium concurrent with drug treatment (Fig. 7). The pattern of labeling of rRNA in cells treated with sangivamycin or toyocamycin under these conditions was the same as that by [^{3}H]Urd (Fig. 4). Neither sangivamycin nor toyocamycin caused a reduction in the methylation of the 45 S rRNA precursor.

Protein synthesis initiation complex formation. The inhibition of protein synthesis by sangivamycin was further studied by measuring the formation of the complexes required for the initiation of translation (Fig. 8). The 43 S preinitiation complex consists of the small ribosomal subunit, initiation factors, Met-tRNA$_f$, and mRNA. The 80 S initiation complex is formed by addition of the large ribosomal subunit to the 43 S complex. Cells were exposed to 1 μM concentrations of each compound for 6 hr (including the 30-min assay time). Under these conditions, sangivamycin inhibited protein synthesis to 40% of control, while toyocamycin inhibited protein synthesis to 80% of control (Fig. 3A). Sangivamycin reduced the formation of the 43 S preinitiation complex and 80 S initiation complex to 28 ± 6 and 33 ± 3% of control,

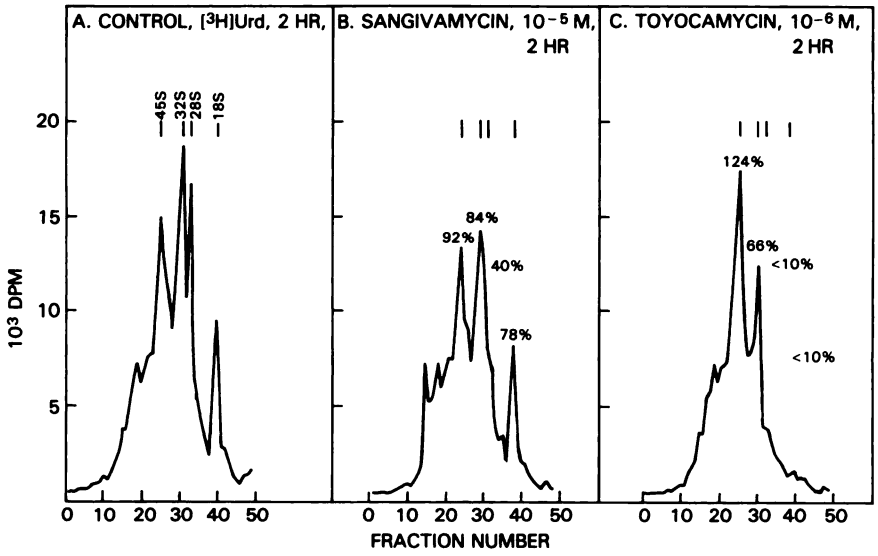

FIG. 4. *Early effects of sangivamycin and toyocamycin on rRNA processing*
Cells were labeled for 2 hr with 1.0 μCi/ml [³H]Urd concurrent with drug treatment. Total RNA was extracted and analyzed as described in Materials and Methods. The results are a representative example of three separate experiments.

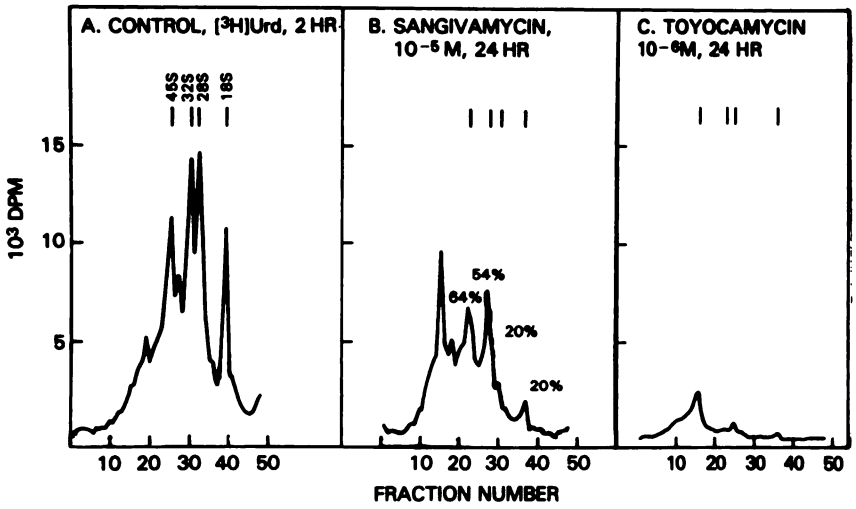

FIG. 5. *Late effects of sangivamycin and toyocamycin on rRNA processing*
Cells were labeled with 1.0 μCi/ml [³H]Urd during the final 2 hr of a 24-hr drug treatment. Total RNA was extracted and analyzed as described in Materials and Methods. The results are an example of two separate experiments.

respectively, while toyocamycin reduced the formation of the complexes to 69 ± 8 and 75 ± 6% of control (Fig. 8). Neither sangivamycin nor toyocamycin significantly affected the specific activity of the Met-tRNA pool as measured by the $^{35}S/^{3}H$ ratio of the tRNA peak. The radioactivity of the tRNA peak is indeed incorporated into tRNA rather than protein as judged by its solubility in hot trichloroacetic acid. These results indicate that sangivamycin inhibits protein synthesis by inhibiting the formation of the 43 S preinitiation complex. The lower 80 S initiation complex level probably reflects the reduction of the 43 S preinitiation complex from which it is formed.

DISCUSSION

The results of this study demonstrate surprisingly different mechanisms of action for the two very closely related pyrrolopyrimidine nucleoside analogs, sangivamycin and toyocamycin. The most conspicuous effect of sangivamycin is the inhibition of protein synthesis. Other compounds that incorporate into RNA and have a similar effect on protein synthesis include 5-azacytidine, 3-deazaguanine, 8-azaguanine, and 9-deazaadenosine (10, 17–21). These drugs also cause a decrease in the formation of protein synthesis initiation complexes like that produced by sangivamycin.

Two hypothetical mechanisms by which sangivamycin inhibits protein synthesis are consistent with these results. First, the incorporation of sangivamycin into mRNA may reduce the ability of mRNA to bind into the 43 S preinitiation complex. Second, less mRNA may be available to bind with the complexes. Total poly(A)-RNA from HT-29 cells treated with 10 μM sangivamycin for 24 hr had 70% of the translational capacity of an equal amount of poly(A)-RNA from untreated cells in a reticulocyte lysate translation system (22). This is far less

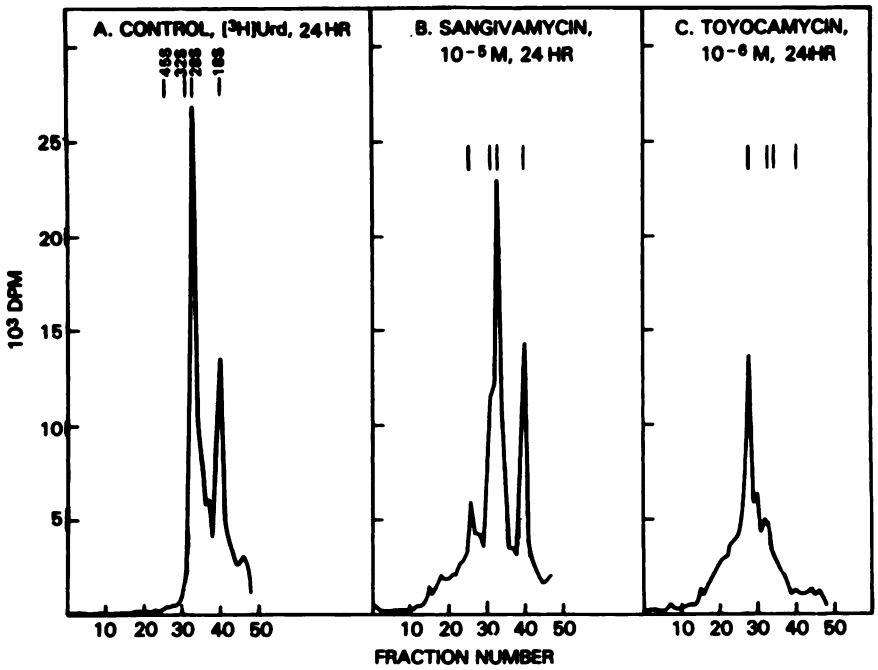

FIG. 6. *Long term effects of sangivamycin and toyocamycin on rRNA processing*

Cells were labeled with 0.2 μCi/ml [^{3}H]Urd for 24 hr concurrent with drug treatment. Total RNA was extracted and analyzed as described in Materials and Methods. The results are a representative example of three separate experiments.

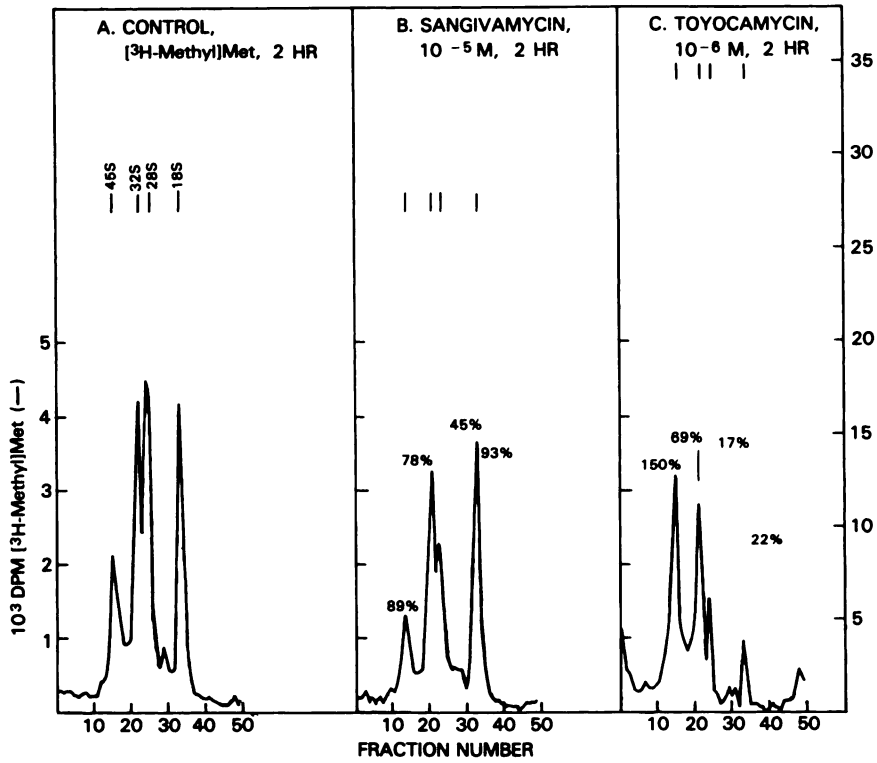

FIG. 7. *Effects of sangivamycin and toyocamycin on methylation of rRNA*

Cells were labeled with 4.0 μCi/ml [*methyl*-^{3}H]methionine for 2 hr concurrent with drug treatment. Total RNA was extracted and analyzed as described in Materials and Methods.

than the inhibition seen *in vitro* after only 6 hr. Therefore, more than just the ability of mRNA to bind to the initiation complex is affected. Perhaps the processing of nuclear heterogeneous RNA to form mature cytoplasmic mRNA is affected. This heterogeneous RNA might not be available for protein synthesis *in vitro*, but may still be able to activate a cell-free translation system. Both of these mechanisms may contribute to protein synthesis inhibition via reduced formation of initiation complexes.

The primary effect of toyocamycin is the inhibition of

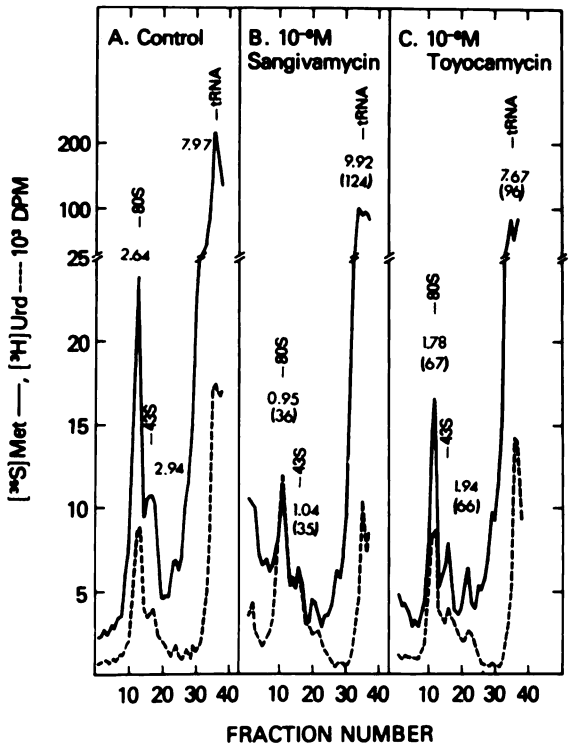

FIG. 8. *Effect of sangivamycin and toyocamycin on formation of protein synthesis initiation complexes*

Cells were prelabeled with [^{3}H]Urd (- - -), incubated with drug for 5.5 hr, and pulse-labeled with [^{35}S]methionine (——). The 10,000 × *g* supernatant was resolved on 10–30% glycerol gradients as described in Materials and Methods. The ratio of ^{35}S to ^{3}H is given for each peak. The ratio as a percentage of control is shown in parentheses for drug-treated cells. The direction of sedimentation is from left to right. The results are a representative example of four separate experiments.

the maturation process of the 45 S ribosomal RNA precursor. This effect also occurs in HeLa, Novikoff hepatoma, and L5178Y cells (8, 14, 15). The initial 45 S rRNA transcript contains sequentially, from the 5' end, an external transcribed spacer, 18 S rRNA, an internal transcribed spacer (ITS 1), 5.8 S rRNA, a second internal transcribed spacer (ITS 2), and 28 S rRNA. The initial transcript is immediately associated with a group of proteins, and all further processing occurs within the framework of this ribonucleoprotein particle (23). A series of endonucleolytic cleavages at specific sites produce characteristic intermediates and finally mature rRNA (24). These characteristic intermediates depend on the cells studied. The most abundant intermediate observed in HT-29 cells is a 32 S fragment containing 5.8 S rRNA, ITS 2, and 28 S rRNA, which also has been observed in the human HeLa cell line (25). Theories on the mechanism by which processing is inhibited include: 1) an inability of the initial transcript to associate properly with required proteins (15, 26), 2) changes in the methylation pattern of rRNA (8), and 3) changes in rRNA conformation induced by analog incorporation such that enzymes which process rRNA are unable to function (26). Studies have not yet found any difference between the protein content of ribonucleoprotein particles formed in the presence or absence of either toyocamycin or 5-azacytidine (15, 26). Also, neither we nor others have found that nucleoside analogs that inhibit 45 S rRNA processing reduce methylation of the 45 S rRNA precursor (8). However, nondenatured 45 S rRNA from cells treated with 5-azacytidine had a different electrophoretic mobility than that from untreated cells, but that difference disappeared under denaturing conditions (26). This result implies, but does not necessarily prove, that drug substitution can cause a change in conformation related to the hydrogen bonding involved in base pairing. There are extensive double-stranded regions in precursor rRNA (27), and denaturation of these regions resulting from incorporation of analogs could disrupt precursor rRNA processing.

Toyocamycin is extremely cytotoxic and the loss of cell viability correlates with the concentration-dependent build-up of 45 S precursor rRNA (28). On the other hand, sangivamycin is much less cytotoxic, even at 100 times the concentration required for inhibition of protein synthesis. It is possible that once unprocessable rRNA precursor accumulates in the nucleolus following treatment with toyocamycin, irreversible damage occurs, and even after drug removal cell death ensues. The inhibition of protein synthesis by sangivamycin may be more easily reversed after drug removal, allowing greater cell survival. These observations have implication for the future use of these compounds.

This report describes a pair of structurally similar compounds each of which acts via incorporation into RNA but appears to have different selectivities. Toyocamycin alters the processing of rRNA precursor, while sangivamycin bypasses this effect and affects protein synthesis. Further characterization on the molecular level of the changes in RNA caused by these two compounds may show how RNA structure relates to function.

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