MOLECULAR PHARMACOLOGY, 20:657–661

Cytokinetic and Biochemical Effects of Sangivamycin in Human Colon Carcinoma Cells in Culture

ROBERT I. GLAZER AND KATHLEEN D. HARTMAN

Applied Pharmacology Section, Laboratory of Medicinal Chemistry and Biology, National Cancer Institute, Bethesda, Maryland 20205

Received March 30, 1981; Accepted June 11, 1981

SUMMARY

The cytotoxic and biochemical activity of the pyrrolopyrimidine antibiotic, sangivamycin, was examined in a human colon carcinoma in tissue culture. Logarithmically growing cells were more sensitive than early plateau-phase cells to the lethal effects of the drug as determined by colony formation in soft agar. Cell lethality in both log- and plateau-phase cells became more pronounced when drug exposure time was increased from 2 hr to 24–48 hr. Reduced cell viability correlated with the inhibition of total RNA synthesis after 2 hr of drug exposure, and with reduced DNA synthesis and incorporation of drug into DNA and RNA after 24–48 hr of drug exposure. Fractionation of total RNA into non-poly(A)-containing and poly(A)-containing RNA indicated that inhibition of the synthesis of both RNA species occurred after 24 hr of exposure of log-phase cells to sangivamycin. Neither RNA fraction was affected in plateau-phase cells after 48 hr of drug exposure. In contrast, a close correlation was found between the incorporation of [^{3}H]sangivamycin into poly(A)RNA and cell lethality after 24–48 hr of exposure of both log- and plateau-phase cells to sangivamycin. These results show that a human colon carcinoma is responsive to sangivamycin following prolonged drug-exposure intervals and that the associated cytotoxicity correlates closely with the incorporation of drug into mRNA, as well as inhibition of DNA synthesis.

INTRODUCTION

The pyrrolopyrimidine antibiotic, sangivamycin, is an adenosine analogue which possesses significant antitumor activity against a variety of animal tumors, including L1210 and P388 leukemias and colon carcinoma 26.[1] In early Phase I clinical trials, mild toxicity was noted but no clinical responses were observed with pulse intravenous administration of the drug (1). In contrast to most adenosine analogues, sangivamycin is not deaminated (2); however, sangivamycin serves as a substrate for adenosine kinase (3) and is incorporated into DNA and RNA of normal tissues (4). As the drug triphosphate, sangivamycin competes with ATP to inhibit RNA polymerase activity (5) and the aminoacylation of tRNA (6), and is incorporated into the terminal sequence of tRNA in a cell-free system *in vitro* (6).

Recent studies with L1210 ascites cells *in vitro* indicated that sangivamycin inhibited nRNA synthesis whereas DNA synthesis and RNA methylation were unaffected after 1 hr of drug exposure (7). In these investigations, no preference for inhibition of a particular species of RNA by sangivamycin was noticed, but the incorporation of ATP into nRNA was preferentially inhibited (7). In cytokinetic studies with sarcoma 180 cells in tissue culture, Ritch *et al.* (8) reported a marked time and concentration dependence for the lethal effect of sangivamycin on proliferating and plateau-phase cells. In these studies, inhibition of RNA and DNA synthesis occurred in proportion to the increased cell lethality produced by the drug. In this instance, the onset of inhibition of RNA synthesis preceded the inhibition of DNA synthesis, although the latter process was eventually inhibited to a greater degree (97% inhibition of DNA synthesis and 70% inhibition of RNA synthesis after exposure of cells to 1×10^{-6} M sangivamycin for 24 hr).

In the present report, we investigated the cytokinetic and biochemical effects of sangivamycin on a human colon carcinoma in tissue culture. These studies confirm the marked time dependence of the lethal effect of sangivamycin on cell viability and indicate that DNA synthesis and the incorporation of drug into poly(A)RNA best correlate with the cytotoxicity produced by this agent.

MATERIALS AND METHODS

Materials. [5-*methyl*-^{3}H]Thymidine (20 Ci/nmole) and [*U*-^{14}C]uridine (522 mCi/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.). Sangivamycin was obtained from the Drug Synthesis and

[1] J. Plowman, personal communication.

0026-895X/81/030657-05$02.00/0

Chemistry Branch, National Cancer Institute (Bethesda, Md.). RPMI 1640 was purchased from Hem Research Inc. (Rockville, Md.), heat-inactivated fetal calf serum from Grand Island Biological Company Laboratories (Grand Island, N. Y.), and gentamycin from Flow Laboratories (McLean, Va.). [^{3}H]Sangivamycin (292 mCi/mmole) was prepared from [^{3}H]toyocamycin (New England Nuclear Corporation) by hydrolysis in 2 N HCl at 100° for 4 hr (9). [^{3}H]Sangivamycin was purified by lyophilization followed by high-performance liquid chromatography using a Whatman Partisil SCX column (0.46 × 25 cm) and isocratic elution with 25 mM KH_2PO_4 (pH 3.65)-5% acetonitrile at a flow rate of 1 ml/min. Sangivamycin eluted in 9 min and toyocamycin eluted in 5 min under these conditions.

*Tissue culture.*HT-29 cells originally derived from a human colon carcinoma (10) were obtained from Dr. L. Erickson, National Cancer Institute. Cells were grown under 5% CO_2-air in RPMI 1640 supplemented with 10% fetal calf serum and gentamycin, 50 μg/ml. Cell inocula comprised 0.83 × 10^5 cells/10 ml of medium in 25-cm^2 plastic flasks.

Drug treatment. Log-phase (3-day) cells or early plateau-phase (5-day) cells were treated with 10^{-7}, 10^{-6}, and 10^{-5} M sangivamycin or [^{3}H]sangivamycin dissolved in dimethylformamide for 2, 24, and 48 hr. Control flasks received an equivalent concentration (0.1%, v/v) of dimethylformamide alone, which was without effect on cell growth, cell viability, and nucleic acid synthesis. In early plateau-phase cells, the medium was changed prior to 24-hr and 48-hr incubations with sangivamycin. After drug treatment, cells were harvested by trypsinization with 0.05% (w/v) trypsin in Hanks' balanced salt solution without Ca^{2+} and Mg^{2+} and containing 0.02 M EDTA. The growth medium was first decanted and cell monolayers were rinsed with 3 ml of trypsin solution followed by incubation for 10 min at 37° with 0.3 ml of trypsin solution. Trypsinization was terminated by the addition of 10 ml of RPMI 1640 containing 10% fetal calf serum and gentamycin, 50 μg/ml.

Cell viability determinations. After trypsinization, cell dilutions of 200, 1,000, and 10,000 cells were prepared in RPMI 1640 containing 10% fetal calf serum and gentamycin, 50 μg/ml. Soft agar cloning was performed as described by Vistica *et al.* (11) except that RPMI 1640 was used. Duplicate 6-cm Petri dishes were plated with 200, 1,000, and 10,000 cells for each drug-treated and control flask. After 14 days, colonies were fixed with 95% ethanol and stained with 0.01% gentian violet in 1% (v/v) acetic acid. Cell viability is expressed as the number of colonies produced by drug-treated cells divided by the number of colonies produced by control cells (corrected for cloning efficiency) × 100. Cloning efficiency ranged from 60% to 90%.

DNA and RNA determinations. Following the addition of sangivamycin, cells were pulse-labeled during the last hour of drug treatment with 1 μCi of [5-*methyl*-^{3}H]TdR[2] and 1 μCi of [^{14}C]UR. After trypsinization, the cells were centrifuged at 400 × *g* for 10 min at 4° and washed once with ice-cold phosphate-buffered saline (5.6 mM Na_2HPO_4:1.1 mM KH_2PO_4:0.154 M NaCl). DNA and RNA were extracted by the addition of 3 ml of 1% (w/v) sodium dodecyl sulfate/0.1 M Tris-HCl (pH 8.0)/0.01 M EDTA followed by 1.5 ml of phenol mixture (phenol/*m*-cresol/water, 7:2:2, v/v/v containing 0.1% 8-hydroxyquinoline) and 1.5 ml of chloroform. After mixing vigorously with a Vortex agitator for 5 min, the emulsion was clarified by centrifugation at 10,000 × *g* for 10 min. The supernatant was removed and precipitated with 3 volumes of 2% (w/v) potassium acetate in 95% ethanol at −20° overnight. RNA and DNA were collected by centrifugation at 10,000 × *g* for 20 min and dissolved in 0.2 ml of water. The sample was divided into two 0.1-ml aliquots. DNA was obtained by incubating one 0.1-ml sample with 1 ml of 0.01 M Tris-HCl (pH 7.4), 0.2 M NaCl, 0.01 M EDTA, 20 μg of RNAase A, and 20 units of RNase T_1 at 37° for 2 hr. RNAwas obtained by incubating the second 0.1-ml sample with 1 ml of 0.01 M Tris-HCl (pH 7.2), 0.5 M NaCl, 0.01 M $MgCl_2$, and 10 μg of DNase I (electrophoretically pure) at 37° for 2 hr. At the end of each incubation, 3 volumes of 2% potassium acetate in 95% ethanol were added and samples were precipitated at −20° overnight. RNA and DNA were collected by

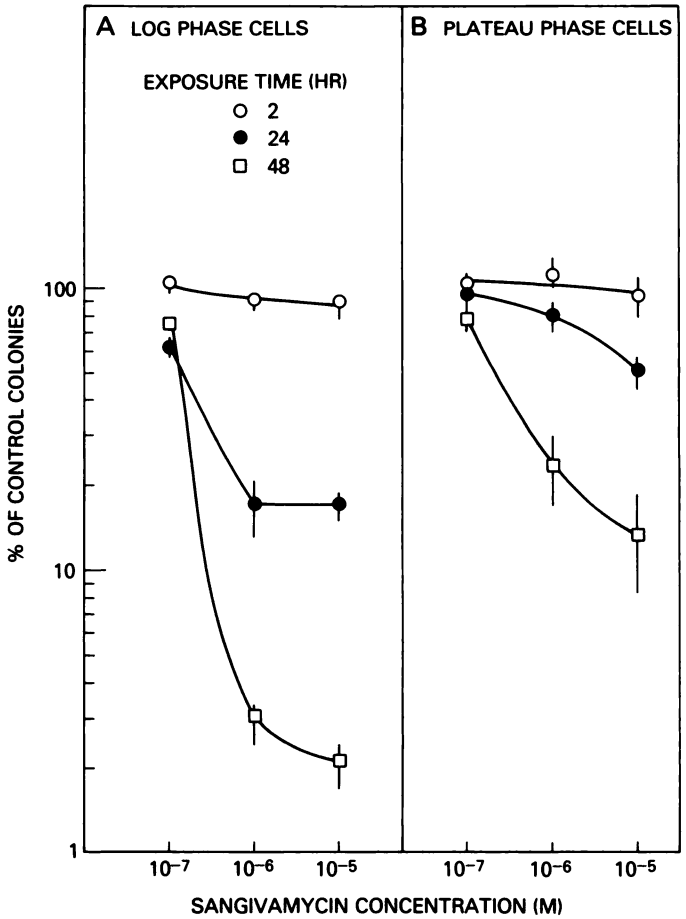

FIG. 1. *Viability of log- and plateau-phase cells after exposure to sangivamycin*

Log- and early plateau-phase cells were exposed for 2, 24, or 48 hr to 10^{-7}, 10^{-6}, and 10^{-5} M sangivamycin. After drug exposure, cells were grown in soft agar, and colony formation was used to determine cell viability. Results are expressed as the percentage of colonies formed from drug-treated cells versus control cells taken as 100%. Each value is the mean ± standard error of four to six determinations.

[2] The abbreviations used are: TdR, thymidine; UR, uridine.

centrifugation at 10,000 × *g* for 20 min and aliquots were removed to determine radioactivity and absorbance at 260 nm.

Poly(A)RNA determination. Cells were labeled during the last hour of drug treatment with 1 μCi of [^{14}C]UR or, alternatively, throughout drug treatment with [^{3}H]sangivamycin, and harvested by trypsinization as described above. The washed cell pellet was extracted with 3 ml of 0.1% sodium dodecyl sulfate, 0.1 M Tris-HCl (pH 9.0), 1.5 ml of phenol mixture, and 1.5 ml of chloroform by mixing vigorously with a Vortex agitator for 5 min. The emulsion was clarified by centrifugation at 10,000 × *g* for 10 min and precipitated with 3 volumes of 95% ethanol at −20° overnight. Total RNA was collected by centrifugation at 10,000 × *g* for 20 min and fractionated into non-poly(A)RNA and poly(A)RNA by poly(U)Sepharose affinity chromatography (12).

RESULTS

Cell viability. Log phase and early plateau phase cells were exposed for 2, 24, and 48 hr to sangivamycin, and cell viability was determined by soft agar cloning (Fig. 1). Brief (2-hr) drug exposure of either log- or plateau-phase cells was not cytotoxic. Prolonging drug exposure to 24 or 48 hr dramatically increased cell lethality; 1 × 10^{-6} M sangivamycin reduced cell viability of log phase cells by 83% and 97% after 24 and 48 hr of drug exposure, respectively, whereas cell viability was reduced in plateau-phase cells by 20% and 76%, respectively. Increasing the concentration of sangivamycin to 1 × 10^{-5} M had little if any effect on increasing cell lethality in log-phase cells, but it did produce a further increase in cytotoxicity in plateau-phase cells.

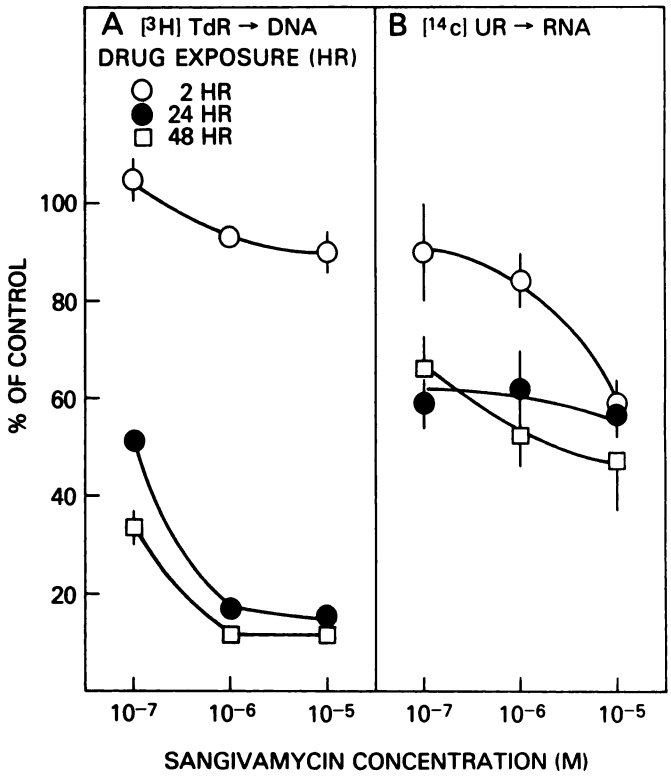

FIG. 2. *Incorporation of [^{3}H]TdR into DNA and [^{14}C]UR into RNA after exposure of log-phase cells to sangivamycin*

Log-phase cells were exposed for 2, 24, and 48 hr to 10^{-7}, 10^{-6}, and 10^{-5} M sangivamycin. During the last hour of drug exposure, cells were incubated with 1 μCi of [^{3}H]TdR and 1μCi [^{14}C]UR, and DNA and RNA were isolated as described under Materials and Methods. Results are expressed as a percentage of the incorporation of either [^{3}H]TdR or [^{14}C]UR into DNA and RNA, respectively, in drug-treated cells versus control cells taken as 100%. Control values (disintegrations per minute/A_{260}) were as follows: 2-hr drug exposure—DNA, 108,400 ± 10,100; RNA, 27,100 ± 2000; 24-hr drug exposure—DNA, 120,900 ± 12,000; RNA 32,500 ± 3,100; 48-hr drug exposure—DNA, 63,300 ± 5,000; RNA, 15,900 ± 1,500. Each value is the mean ± standard error of four to six determinations.

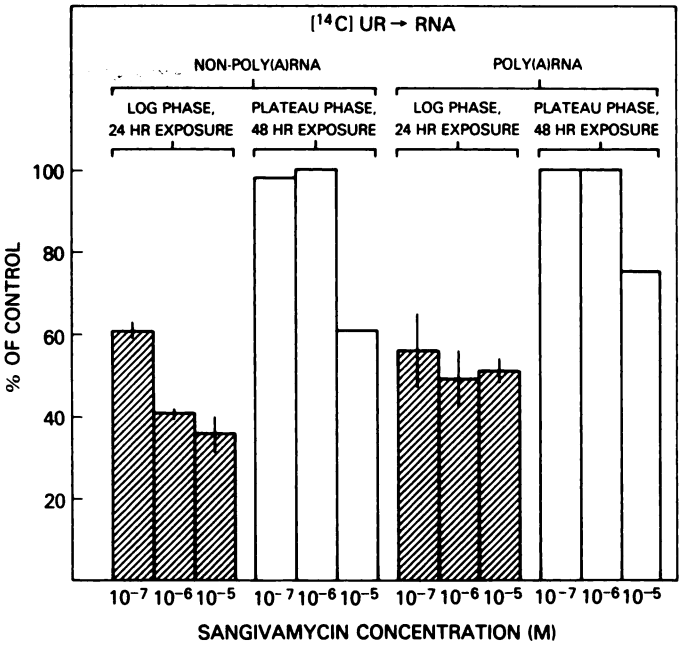

FIG. 3. *Incorporation of [^{14}C]UR into non-poly(A)RNA and poly(A)RNA after exposure of log and plateau-phase cells to sangivamycin*

Log-phase cells were exposed for 24 hr and plateau-phase cells for 48 hr to 10^{-7}, 10^{-6}, and 10^{-5} M sangivamycin. During the last hour of drug exposure, cells were incubated with 1 μCi of [^{14}C]UR and total RNA was isolated and fractionated by poly(U)Sepharose chromatography as described under Materials and Methods. Results are expressed as a percentage of control values taken as 100%. Control values (disintegrations per minute/A_{260}) were as follows: log phase cells—non-poly(A)RNA, 86,300 ± 7800; poly(A)RNA, 177,100 ± 203,000; plateau phase cells—non-poly(A)RNA, 9100 ± 800; poly(A)RNA, 23,600 ± 2100. Each value is the mean of two determinations or the mean ± standard error of four determinations.

DNA and RNA synthesis. To investigate further what parameters might be responsible for the cytotoxicity produced by sangivamycin, DNA and RNA synthesis was measured in log-phase cells under conditions of drug exposure identical with those used in the cell viability studies (Fig. 2). DNA synthesis was not significantly affected after 2 hr of drug exposure, but precipitously decreased following prolonged incubation with sangivamycin for 24 or 48 hr. In contrast, total RNA synthesis was initially inhibited up to 40% after 2 hr of exposure to 1 × 10^{-5} M sangivamycin, but was not reduced much further after longer intervals of drug treatment.

In order to assess whether a specific class of RNA was inhibited more extensively than total RNA by sangiva-

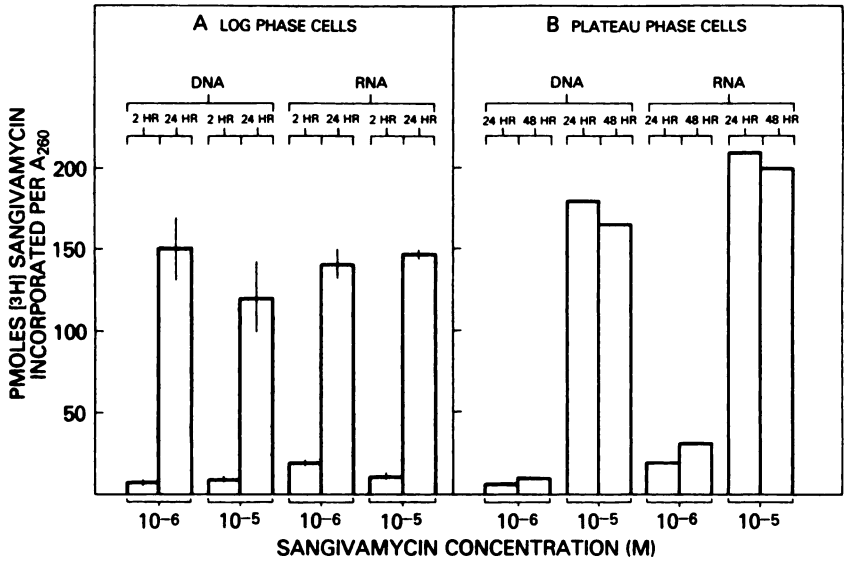

FIG. 4. *Incorporation of [^{3}H]sangivamycin into DNA and RNA of log- and plateau-phase cells*

Log-phase cells were exposed for 2 and 24 hr and plateau-phase cells for 24 and 48 hr to 10^{-6} and 10^{-5} M [^{3}H]sangivamycin. Total RNA and DNA were extracted as described under Materials and Methods. Each value is the mean of two determinations or the mean ± standard error of three determinations.

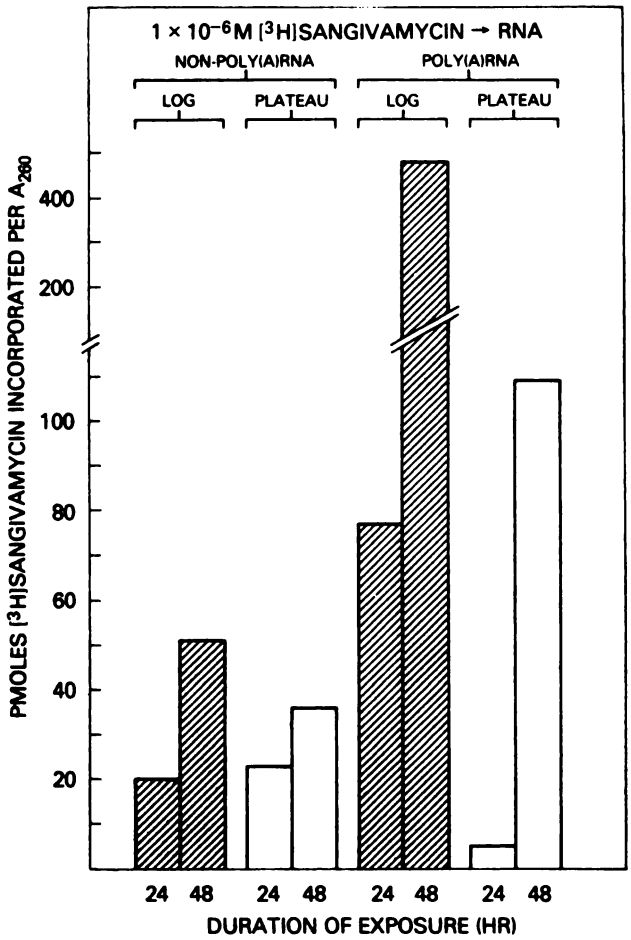

FIG. 5. *Incorporation of [^{3}H]sangivamycin into non-poly(A)RNA and poly(A)RNA of log- and plateau-phase cells*

Log- and plateau-phase cells were exposed for 24 and 48 hr to 10^{-6} M [^{3}H]sangivamycin, and total RNA was isolated and fractionated by poly(U)Sepharose chromatography as described under Materials and Methods. Each value is the mean of two determinations.

mycin, RNA was fractionated into non-poly(A)RNA and poly(A)RNA species (Fig. 3). RNA synthesis was not significantly inhibited in plateau-phase cells after 48 hr of drug exposure, a period wherein a significant decrease in cell viability occurred (Fig. 1). In contrast, inhibition of non-poly(A)RNA and poly(A)RNA occurred to a degree comparable to that of total RNA in log-phase cells (Fig. 2).

[^{3}H]Sangivamycin incorporation into DNA and RNA. Since sangivamycin preferentially inhibited DNA synthesis after 24–48 hr of drug exposure but affected RNA synthesis as early as after 2 hr, we determined whether these phenomena might be related to the incorporation of the drug into DNA and RNA after varying intervals of drug exposure (Fig. 4). Sangivamycin at 1×10^{-5} M was incorporated equally into DNA and RNA in both log- and plateau-phase cells after 24 hr of drug exposure; however, 1×10^{-6} M sangivamycin was incorporated into DNA and RNA more extensively in log-phase cells than in plateau-phase cells after a similar interval. The time and concentration dependence of drug incorporation into DNA and RNA correlated qualitatively with the effect of sangivamycin on cell viability (Fig. 1).

To determine further the significance of the incorporation of [^{3}H]sangivamycin into RNA, determinations were made of its incorporation into total non-poly(A)RNA and poly(A)RNA in log- and plateau-phase cells after 24 and 48 hr of drug exposure (Fig. 5). [^{3}H] Sangivamycin was incorporated more extensively into poly(A)RNA in log-phase cells than in plateau-phase cells. In addition, a greater incorporation of drug into poly(A)RNA than into non-poly(A)RNA occurred in log-phase cells as well as in plateau-phase cells after 48 hr of drug exposure. The incorporation of [^{3}H]sangivamycin into poly(A)RNA also correlated with the reduction in cell viability observed in log- and plateau-phase cells under comparable conditions.

DISCUSSION

The present study indicates that sangivamycin produces significant cell lethality to a human colon carcinoma which is generally refractory to antitumor agents such as the nitrosoureas (13). One striking characteristic of the cytokinetics of cell lethality is the exponential plateau survival curve (Fig. 1). Survival patterns such as these are reminiscent of those of fluoropyrimidines, which become self-limiting beyond a certain cytotoxic concentration (14). Since sangivamycin inhibited DNA synthesis in proportion to its cytocidal activity, an increasingly slower cell cycle traverse would be expected to produce a progressively lesser effect on cell lethality.

The long drug-exposure intervals required for sangivamycin to produce cytotoxicity suggests that the drug must first be anabolized and incorporated into nucleic acids. This hypothesis is substantiated by the fact that the drug is incorporated into DNA and RNA in a concentration- and time-dependent fashion similar to that required to produce cell lethality.

An additional parameter to consider is the early but limited inhibition of RNA synthesis. Inhibition of total RNA synthesis reached a plateau with increasing drug concentration and duration of drug exposure. Although this effect did not correlate closely with increased cell lethality, the incorporation of sangivamycin into total RNA, and particularly into poly(A)RNA, did show a strong association with cytotoxicity in both log- and plateau-phase cells. The fact that poly(A)RNA synthesis was not inhibited by sangivamycin suggests that the high rate of incorporation of drug into this species of RNA may be specifically related to its cytotoxic action. Similar results have also been obtained in sarcoma 180 cells in culture (15). Although the precise relationship between incorporation of sangivamycin into poly(A)RNA and inhibition of DNA synthesis has not been defined, it is possible that drug-substituted mRNA interferes with translation, which in turn results in a slower progression of cells through S phase with concomitant inhibition of DNA synthesis.

REFERENCES

1. Cavins, J. A., T. C. Hall, K. B. Olson, C. L. Khung, J. Horton, J. Colsky, and R. A. Shadduck. Initial toxicity study of sangivamycin (NSC 65346). *Cancer Chemother. Rep.* **51**:197–200 (1967).
2. Suhadolnik, R. J. Pyrrolopyrimidine antibiotics, in *Nucleoside Antibiotics* (R. J. Suhadolnik, ed.), Vol. 1. Wiley-Interscience, New York, 298–353 (1970).
3. Hardesty, C. T., N. A. Chaney, V. S. Waravdekar, and J. A. R. Mead. Enzymatic phosphorylation of sangivamycin. *Biochim. Biophys. Acta* **195**: 581–583 (1969).
4. C. T. Hardesty, N. A. Chaney, V. S. Waravdekar, and J. A. R. Mead. The disposition of the antitumor agent, sangivamycin, in mice. *Cancer Res* **34**: 1005–1009 (1974).
5. R. J. Suhadolnik, T. Uematsu, and H. Uematsu. The incorporation of sangivamycin 5′-triphosphate into polyribonucleotide by ribonucleic acid polymerase in *Micrococcus lysodeikticus*. *J. Biol. Chem.* **243**:2761–2766 (1968).
6. S. C. Uretsky, G. Acs, E. Reich, M. Mori, and L. Altwerger. Pyrolopyrimidine nucleotides and protein synthesis. *J. Biol. Chem.* **243**:306–312 (1968).
7. Glazer, R. I., and A. L. Peale. Comparison between the inhibitory activities of sangivamycin and thiosangivamycin on nuclear RNA synthesis in L1210 cells *in vitro*. *Biochem. Pharmacol.* **29**:305–310 (1980).
8. Ritch, P. S., R. I. Glazer, R. E. Cunningham, and S. E. Shackney. Kinetic effects of sangivamycin in sarcoma 180 cells *in vitro*. *Cancer Res.* **41**:1784–1788 (1981).
9. Rao, K. V. Structure of sangivamycin. *J. Med. Chem.* **11**:939–941 (1968).
10. Fogh, J., and G. Trempe. New human tumor cell lines, in *Human Tumor Cells in Vitro* (J. Fogh, ed.). Plenum Press, New York, 115–159 (1975).
11. Vistica, D. T., J. N. Toal, and M. Rabinovitz. Amino acid-conferred resistance to melphalan. I. Structure-activity relationship in cultured murine L1210 leukemia cells. *Cancer Treat. Rep.* **60**:1363–1368 (1976).
12. Glazer, R. I., and K. D. Hartman. Analysis of the effect of 5-fluorouracil on the synthesis and translation of poly(A)RNA from Ehrlich ascites cells. *Mol. Pharmacol.* **19**:117–121 (1981).
13. Erickson, L. C., R. Osieka, and K. W. Kohn. Differential repair of 1-(2-chloroethyl)-3-(4-methylcyclohexyl)-nitrosourea-induced DNA damage in two human colon tumor cell lines. *Cancer Res.* **38**:802–808 (1978).
14. Drewinko, B., L. Y. Yang, D. H. W. Ho, J. Benevenuto, T. L. Loo, and E. J. Freireich. Treatment of cultured human colon carcinoma cells with fluorinated pyrimidines. *Cancer* **45**:1144–1158 (1980).
15. Ritch, P. S., and R. I. Glazer. Preferential incorporation of sangivamycin into RNA in sarcoma 180 cells *in vitro*. *Biochem.* Pharmacol., in press.

Send reprint requests to: Dr. Robert I. Glazer, National Cancer Institute, Building 37, Room 6D28, Bethesda, Md. 20205.