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Inhibition of Histone H1 Phosphorylation by Sangivamycin and other Pyrrolopyrimidine Analogues

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SUMMARY

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We have examined the ability of the pyrrolopyrimidine antibiotic, sangivamycin [4-amino-5-carboxamide-7(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine], to inhibit the phosphorylation of the 2% trichloroacetic acid-soluble nuclear proteins from Ehrlich ascites cells *in vitro*. In whole cells, sangivamycin inhibited histone H1 phosphorylation, with 50% inhibition observed at a drug concentration of 100 μM, but phosphorylation of the high-mobility group (HMG) proteins, HMG 14 and 17, was unaffected. At all concentrations, histone H1 phosphorylation was inhibited to a greater degree than nRNA synthesis. The pyrrolopyrimidine analogues toyocamycin, sangivamycin-amidine, and sangivamycin-amidoxime did not differ substantially from sangivamycin in their inhibitory effects on histone H1 phosphorylation and nRNA synthesis in whole cells, whereas thiosangivamycin was approximately 50-fold more potent. Cell-free assays with partially purified nuclear protein kinase activities, PK-I and PK-II, revealed that sangivamycin was a competitive inhibitor versus ATP with either histone H1 or casein as substrate. The 50% inhibitory concentration of thiosangivamycin for PK-I activity was 40-fold less than sangivamycin and closely paralleled their relative inhibitory activities for inhibiting histone H1 phosphorylation in intact cells *in vitro*. This relationship was not apparent for sangivamycin-amidine, sangivamycin-amidoxime, and toyocamycin.

INTRODUCTION

Phosphorylation of histone and nonhistone nuclear proteins has been correlated with several aspects of transcriptional control in eukaryotes. Inhibition of this post-synthetic modification of nuclear proteins by nucleoside analogues has been demonstrated for several systems and may contribute to the inhibition of transcription by these drugs. Cordycepin (3′-deoxyadenosine) is a competitive inhibitor of cyclic nucleotide-dependent and -independent protein kinases from various sources (1). Cordycepin and cordycepin triphosphate inhibited the phosphorylation of urea-soluble nuclear proteins of rat liver (2), and cordycepin and xylosyladenine inhibited nuclear protein phosphorylation in L1210 cells, particularly in the presence of the adenosine deaminase inhibitor, deoxycoformycin (3). In view of the latter observation, it could be expected that a nucleoside that is not deaminated would be an effective inhibitor of protein phosphorylation.

Sangivamycin (4-amino-5-carboxamide-7(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine) (4) is a naturally occurring pyrrolopyrimidine antibiotic produced by *Streptomyces rimosus* and is structurally related to adenosine (Fig. 1). Sangivamycin demonstrates significant antitumor activity against L1210 leukemia in mice (5) and has been of clinical interest (6). The antibiotic can be phosphorylated to the drug triphosphate by mouse liver extracts (7) and is a substrate for ribonucleotide reductase (8), but is neither a substrate nor inhibitor of adenosine deaminase (7). Sangivamycin also inhibits *de novo* synthesis of purine nucleotides, but this is not a primary site of action since addition of purines or pyrimidines does not prevent or reverse the cytotoxicity (9). Sangivamycin triphosphate can compete with ATP in various polymerization reactions. It is incorporated into RNA by *Micrococcus lysodeikticus* RNA polymerase, transcribing calf thymus DNA (10), and into the RNA of several mouse tissues (11). The drug triphosphate can also substitute for ATP with tRNA pyrophosphorylase, leading to its incorporation into the terminal sequence of tRNA and subsequent inhibition of aminoacylation (12). Inhibition of nRNA, but not DNA, synthesis is observed in drug-treated L1210 cells (13). However, the processes which lead to reduced RNA synthesis are not fully understood.

In this report, we examine the ability of sangivamycin

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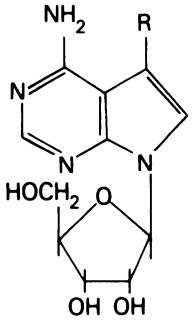

Compound	R
Sangivamycin	O ‖ -C-NH_2
Sangivamycin-amidine	NH ‖ -C-NH_2
Sangivamycin-amidoxime	NOH ‖ -C-NH_2
Thiosangivamycin	S ‖ -C-NH_2
Toyocamycin	-C ≡ N

FIG. 1. *The chemical structures of the pyrrolopyrimidine nucleosides*

and its analogues to inhibit nuclear protein phosphorylation and nRNA synthesis. In view of the large number and complex nature of the nuclear phosphoproteins, we have chosen to examine the phosphorylation of a very limited class of proteins for which structural or regulatory functions are known. This group of proteins, the 2% trichloroacetic acid-soluble nuclear proteins, includes histone H1 and the HMG[1] proteins, of which HMG 14 and 17 can be phosphorylated (14).

MATERIALS AND METHODS

Materials. The amidine and amidoxine analogues of sangivamycin were the generous gift of Dr. R. K. Robins, Brigham Young University (15). All other drugs were obtained from the Drug Synthesis and Chemistry Branch, National Cancer Institute. [^{32}P]Orthophosphoric acid in HCl-free water and [γ-^{32}P]ATP (26 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass.).

Whole cell incubations. Ehrlich ascites cells, maintained i.p. in Swiss mice, were isolated and incubated basically as described (14). Briefly stated, 5×10^7 cells were suspended in 12.5 ml of RPMI 1630 medium without phosphate but supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.4) and 10% newborn calf serum. Unless stated otherwise, cells were incubated at 37° in the presence of drug for 15 min prior to an additional 2-hr incubation with 1 mCi of $^{32}P_i$. Sangivamycin and its derivatives were dissolved in dimethylformamide and an equal amount of dimethylformamide (0.2% final concentration) was added to controls. This concentration of dimethylformamide produced no detectable change in nuclear protein phosphorylation or RNA synthesis.

After cell incubations, nuclei were isolated by Triton X-100 lysis followed by centrifugation through a 0.34/0.88 M sucrose step gradient containing 5 mM $MgCl_2$ (16) and extracted with 2% TCA. These TCA-soluble proteins were precipitated with 25% TCA, washed with cold acetone, dried, and analyzed on acetic acid-urea polyacrylamide gels as described by Panyim and Chalkley (17). Incorporation of $^{32}P_i$ into specific protein species was quantitated by scintillation counting of individual Coomassie blue-stained bands. Gels were also photographed, and a densitometer tracing of the negative was made in order to correct for slightly differing amounts of protein loaded onto the gel.

Nuclear RNA was extracted from the 2% TCA-insoluble nuclear material with 0.1% sodium dodecyl sulfate-0.1 M Tris-HCl (pH 9.0) by vigorous Vortexing followed by deproteinization with phenol-cresol-water (7:2:2, v/v) containing 0.1% 8-hydroxyquinoline.

Incubation of nuclei. Isolated nuclei were resuspended in 0.25 M sucrose-25% glycerol-50 mM Tris-20 mM magnesium acetate (pH 8) to 20 A_{260} units/ml, and 10 μCi of [γ-^{32}P]ATP (26 Ci/mmole) were added. After 30 min at 37°, nuclei were pelleted, washed twice with ice-cold buffer, and extracted with 2% TCA as above.

Protein kinase isolation and assay. Protein kinase activities, PK-I and PK-II, were isolated from Ehrlich ascites cell nuclei by the method of Glazer and Morris (18). The protein kinase assay (18) was modified to contain 0.3 ml of 50 mM Tris-HCl (pH 8.0), 20 mM magnesium acetate, either 50 μg of casein or 50 μg of histone H1, and varying amounts of [γ-^{32}P]ATP and drug, and incubated at 25°. One unit of activity is defined as the amount of enzyme that transfers 1 pmole of ^{32}P from [γ-^{32}P]ATP to recovered casein or histone in 4 min under the assay conditions.

RESULTS

Inhibition of protein phosphorylation in cells. A typical analysis of the 2% TCA extract of nuclei on a 16% acrylamide acetic acid-urea gel is shown in Fig. 2. This extract includes histone H1, HMG proteins 1, 2, 14, and 17, and several other unidentified proteins. The principal phosphoproteins are H1, HMG 14, and HMG 17, and a high specific activity protein of M_r ~30,000 just above HMG 1 and HMG 2. The inhibition of phosphorylation of histone H1 was about 50% at 100 μM sangivamycin, but labeling of the other species with $^{32}P_i$ was not detectably inhibited. As shown in Fig. 3, incorporation of label into each protein and nRNA continued to increase

[1] The abbreviations used are: HMG, high mobility group protein; RPMI 1630 medium, Roswell Park Memorial Institute tissue culture medium 1630; sangivamycin, 4-amino-5-carboxamide-7(β-D-ribofuranosyl)pyrrolo[2,3-*d*]-pyrimidine; sangivamycin-amidine, 4-amino-5-carboxamidine-7(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine; sangivamycin-amidoxine, 4-amino-5-carboxamidoxine-7(β-D-ribofuranosyl)-pyrrolo[2,3-*d*]pyrimidine; thiosangivamycin, 4-amino-5-thiocarboxamide-7(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine; toyocamycin, 4-amino-5-cyano-7(β-D-ribofuranosyl)pyrrolo[2,3-*d*]pyrimidine; TCA, trichloroacetic acid.

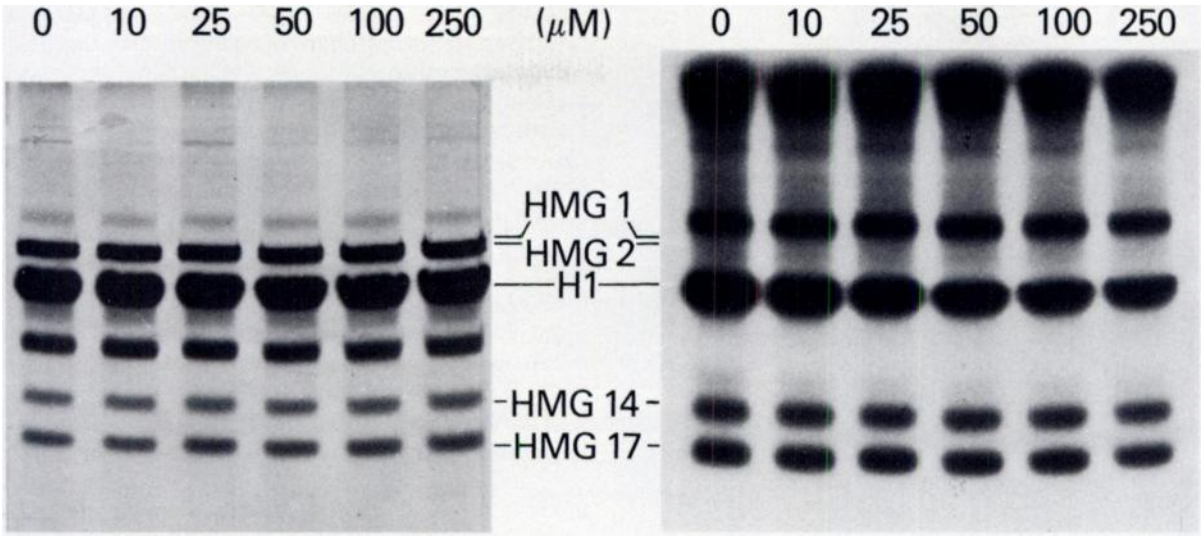

FIG. 2. *Electrophoresis of the 2% TCA-soluble nuclear proteins in 16% acrylamide acid-urea gels*

Ehrlich ascites cells were incubated for 15 min (as described under Materials and Methods) in phosphate-free RPMI 1630 medium in the presence of the indicated concentrations of sangivamycin. $^{32}P_i$ (1 mCi) was added and incubation continued for 2 hr. Nuclei were then isolated and extracted with 2% TCA. After electrophoresis of equal amounts of proteins, the gels were dried and autoradiographed. *Left*, a Coomassie blue-stained gel showing the location of histone H1 and the HMG proteins; *right*, *autoradiographs* of the same gel.

over a 4-hr period. Drug-treated cells also exhibited this increase, but at a reduced rate for histone H1 and nRNA. Inhibition, expressed as a percentage of control, for histone H1, HMG 14, HMG 17, and nRNA is shown in Fig. 4. The effect of various drug concentrations on protein phosphorylation and nRNA synthesis is summarized in Fig. 5. At all concentrations of sangivamycin, inhibition of H1 phosphorylation was greater than inhibition of nRNA synthesis. The IC_{50} values for H1 phosphorylation and nRNA synthesis were approximately 100 and 400 μM, respectively. A much larger drug concentration was required to achieve substantial inhibition of HMG 14 and HMG 17 phosphorylation, for which the IC_{50} was 2–4 mM.

The ability of other drugs structurally related to sangivamycin to inhibit the phosphorylation of histone H1 and HMG 14 and HMG 17 was examined. Toyocamycin is the 5-cyano analogue of the parent pyrrolopyrimidine,

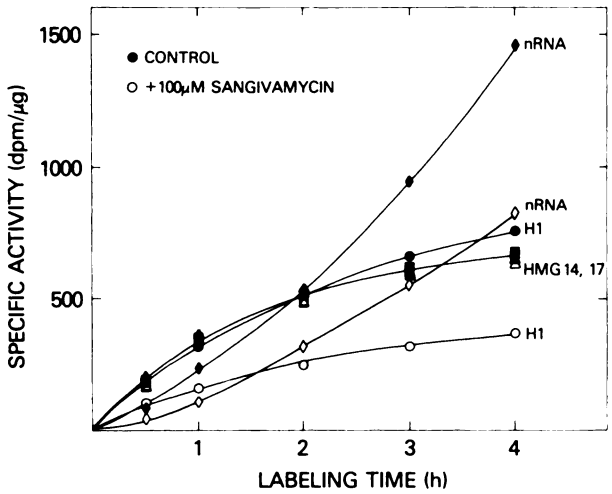

FIG. 3. *Incorporation of ^{32}P label into histone H1, HMG 14, HMG 17, and nRNA in the presence or absence of sangivamycin*

Cells were incubated and incorporation of label was quantitated as described in legend to Fig. 2. The total radioactivity measured was on the order of 10^4 dpm while background was less than 50 dpm. *Closed symbols* represent controls, and *open symbols* represent incubations containing 100 μM sangivamycin (histone H1, ●, ○; HMG 14, ■, □; HMG 17, ▲, △; and nRNA, ◆, ◇).

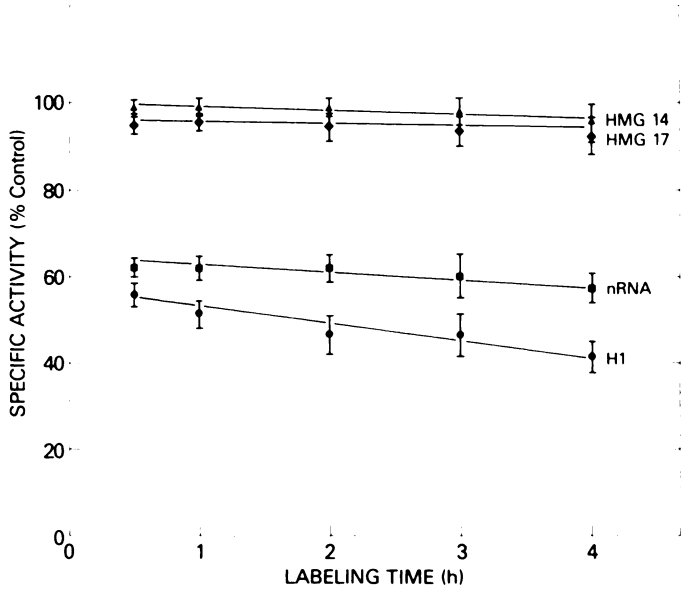

FIG. 4. *Specific activities of histone H1, HMG 14, HMG 17, and nRNA versus labeling time*

Results are expressed as percentage of control incubations without 100 μM sangivamycin. Cells were incubated as described in legend to Fig. 2.

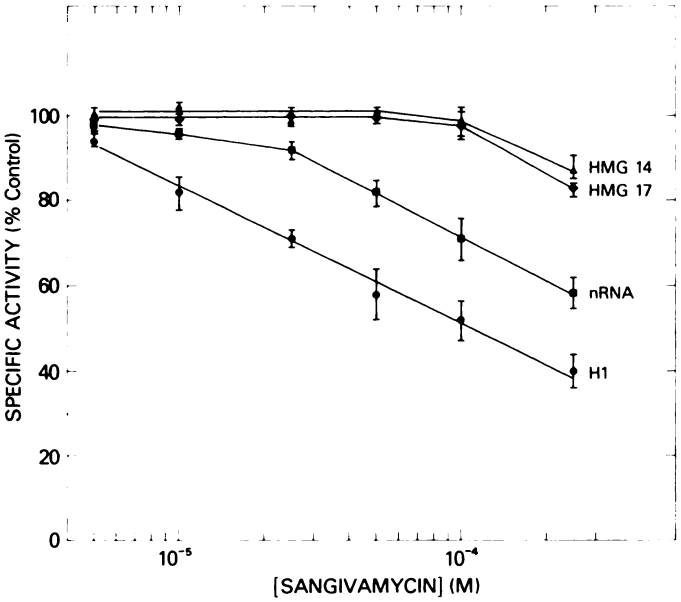

FIG. 5. *The effect of various concentrations of sangivamycin on the phosphorylation of histone H1, HMG 14, and HMG 17 and on the synthesis of nRNA*

Cells were incubated as described in legend to Fig. 2 in the presence of sangivamycin at the indicated concentrations.

7-deazaadenosine, and sangivamycin-amidine, sangivamycin-amidoxime, and thiosangivamycin are the 5-carboxamidine, 5-carboxamidoxime, and 5-thiocarboxamide derivatives (15). The results are summarized in Table 1. With the exception of thiosangivamycin, histone H1 phosphorylation and nRNA synthesis were inhibited approximately to the same degree by equimolar amounts of these drugs as compared with sangivamycin. Similarly, incorporation of label into HMG 14 and HMG 17 was not affected. An equimolar concentration of thiosangivamycin was a more potent inhibitor of histone H1 phosphorylation, and some inhibition of phosphorylation of HMG 14 and 17 was also observed. At a concentration of 10 μM, thiosangivamycin did not inhibit HMG phosphorylation, whereas incorporation of label into histone H1 was still only 24% of control. For the processes examined, thiosangivamycin was approximately 50 times more potent than the other pyrrolopyrimidine analogues. As with sangivamycin, the inhibition of nRNA synthesis by these analogues was not as great as the inhibition of histone H1 phosphorylation. In contrast, actinomycin D substantially inhibited RNA synthesis without affecting protein phosphorylation, and 100 μM adenosine had no effect on either process (data not shown).

Inhibition of protein phosphorylation in cell-free systems. In isolated nuclei, the incorporation of label from [γ-^{32}P]ATP into total nuclear proteins was reduced by approximately 60% in the presence of 100 μM sangivamycin as compared with a similar incubation without the drug (Fig. 6). Note that the pattern of phosphorylation was substantially different for the isolated nuclei in comparison with nuclei isolated from cells incubated with $^{32}P_i$ despite similar intensities of staining of all of the nuclear proteins with Coomassie blue. Of particular interest is the marked decrease in histone H1 phosphorylation in this cell-free system. Variations in the incubation buffer up to 100 mM NaCl did not affect this pattern of protein phosphorylation. In this system, inhibition of phosphorylation of HMG 14 and HMG 17 by 100 μM sangivamycin was observed in the isolated nuclei, whereas at least a 10-fold higher concentration of nucleoside was required for a similar inhibition in intact cells.

As an adenosine analogue, it was expected that sangivamycin would be competitive with ATP. To demonstrate the inhibition kinetics and the general inhibitory nature of sangivamycin, two nuclear protein kinase activities, PK-I and PK-II, were isolated from Ehrlich ascites cells, and the kinetics of inhibition by sangivamycin was assayed by the methods previously described (18). The percentage inhibition by 70 μM sangivamycin was constant for 30 min at 25°, as shown in Fig. 7. The inhibition of the isolated kinase activities with casein as a substrate was competitive, with K_i values of 200 and 100 μM for PK-I and PK-II, respectively (Fig. 8). PK-I and PK-II were equally active with histone H1 as a substrate under identical low ionic strength assay conditions, and identical kinetics and level of inhibition were observed with sangivamycin (data not shown). A comparison of inhibitory activities of the pyrrolopyrimidine analogues in the

TABLE 1

The effect of pyrrolopyrimidine analogues and actinomycin D on the phosphorylation of histone H1, HMG 14 and 17, and nRNA synthesis[a]

Drug	^{32}P Incorporation[b]			
	H1	HMG 14	HMG 17	nRNA
	% control			
Sangivamycin, 100 μM	48 ± 3	97 ± 3	99 ± 1	67 ± 1
Sangivamycin-amidine, 100 μM	56 ± 1	98 ± 6	104 ± 2	84 ± 1
Sangivamycin-amidoxime, 100 μM	53 ± 3	92 ± 11	96 ± 4	88 ± 1
Toyocamycin, 100 μM	45	98	94	64
Thiosangivamycin				
100 μM	10 ± 1	71 ± 4	69 ± 1	38 ± 1
10 μM	24 ± 1	96 ± 4	98 ± 4	60 ± 3
1 μM	93 ± 4	107 ± 5	102 ± 5	95 ± 4
Actinomycin, 1 μM	99	100	99	13

[a] Ehrlich ascites cells were incubated at a density of 4×10^6 cells/ml of RPMI 1630 medium without phosphate but supplemented with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 10% newborn calf serum, and the drug indicated. After 15 min at 37°, [^{32}P]phosphate was added and incubation continued for 2 hr, at which time nuclei were purified and the above components were isolated as described.

[b] Values are means ± standard deviation of three to six experiments or the mean of two experiments.

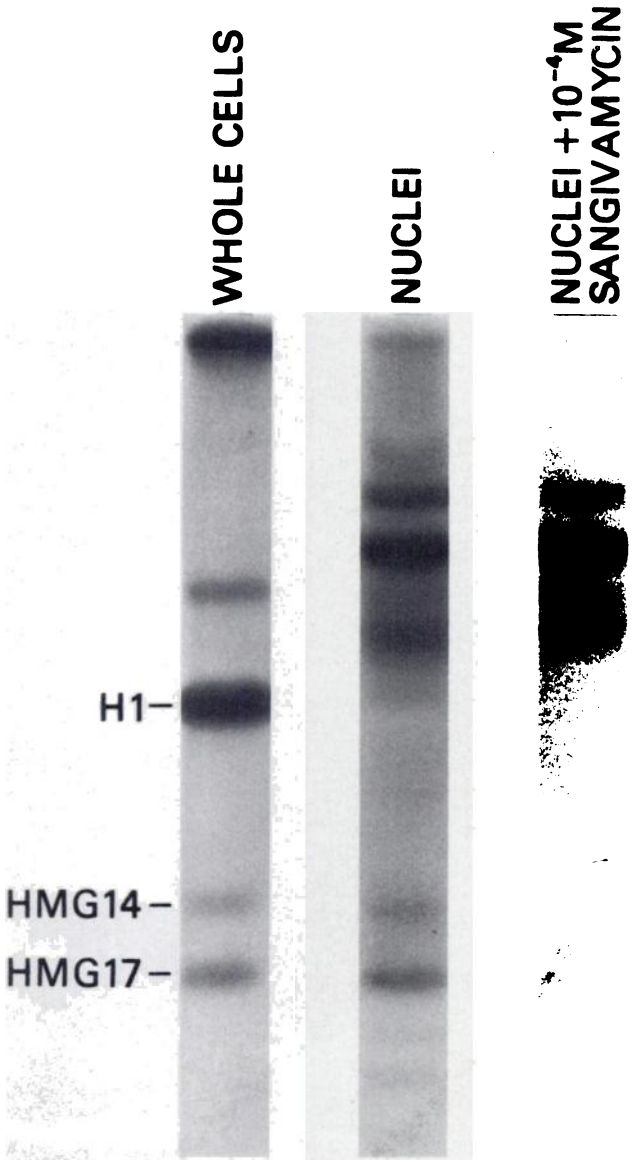

FIG. 6. *Autoradiograms of the 2% TCA-soluble nuclear proteins from isolated nuclei labeled with [γ-^{32}P]ATP in the presence and absence of 100 μM sangivamycin*

For equal amounts of protein, the control track contained 16,400 dpm and the drug-treated sample contained 6,900 (42% control). For comparison, the phosphorylation of the 2% TCA-soluble nuclear proteins labeled with $^{32}P_i$ in intact cells is shown on the *left*.

kinase assays is shown in Table 2. It is noteworthy that thiosangivamycin was 40-fold more inhibitory toward PK-I with histone as a substrate, a result similar to that obtained with intact cells (Table 1). Inhibition of either PK-I or PK-II by the other analogues did not closely parallel their inhibitory effects on histone H1 phosphorylation in intact cells *in vitro*.

DISCUSSION

The results described suggest that sangivamycin and its analogues may produce their cytotoxic activity, in part, by the inhibition of nuclear protein kinases, which

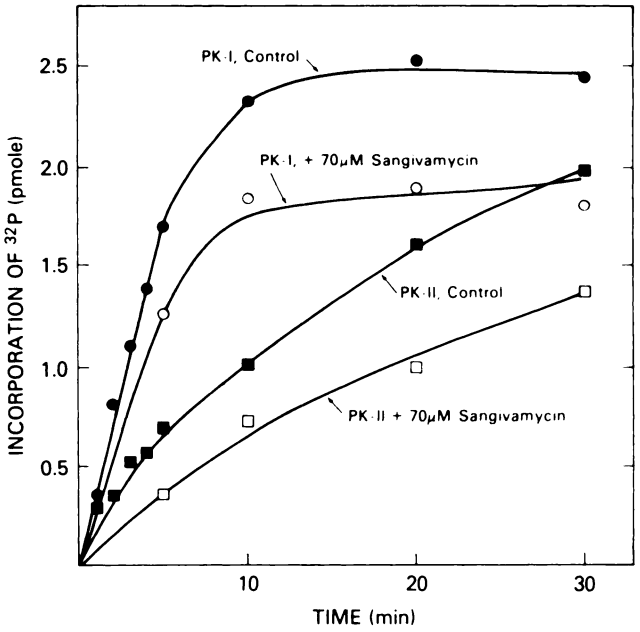

FIG. 7. *Inhibition of protein kinases PK-I and PK-II from Ehrlich ascites cells by sangivamycin*

The protein kinase assays were carried out as described under Materials and Methods using 2 μCi of [γ-^{32}P]ATP (26 Ci/mmole). After incubation for various times, substrate was recovered as acid-insoluble material. *Closed* (●) and *open* (○) *symbols* represent assays run in the absence or presence of 70 μM sangivamycin, respectively.

in turn leads to the observed decrease in protein phosphorylation. The IC_{50} for sangivamycin was approximately the same as the dose required to reduce the clonogenicity of S-180 cells by 60% during a 1-hr drug exposure (19) when corrected for differences in cell densities at the time of drug treatment. Histone H1 phosphorylation, shown here to be more easily inhibited by

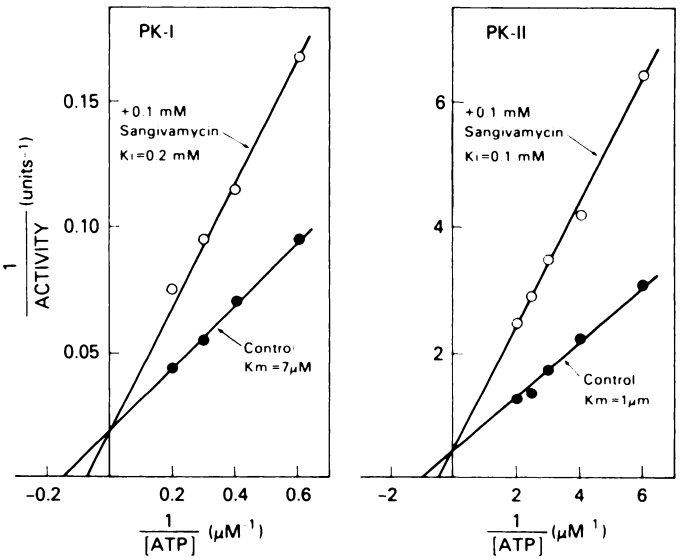

FIG. 8. *Kinetics of inhibition by sangivamycin of the nuclear protein kinases PK-I and PK-II from Ehrlich ascites cells*

The assays were carried out as described in Fig. 7 using varying amounts of [γ-^{32}P]ATP, and incubations were carried out for 4 min in the presence or absence of 100 μM sangivamycin.

TABLE 2

The effect of pyrrolopyrimidine analogues on the isolated protein kinase activities, PK-I and PK-II[a]

Drug	IC_{50}	
	PK-I	PK-II
	μM	
Sangivamycin	200	100
Thiosangivamycin	5 (40)	200 (0.5)
Sangivamycin-amidine	100 (2)	100 (1)
Sangivamycin-amidoxime	60 (3)	600 (0.2)
Toyocamycin	15 (13)	120 (0.8)

[a] The IC_{50} of each drug was determined from assays of the protein kinase activities as described under Materials and Methods, using histone H1 as a substrate and various concentrations of each drug. The values in parentheses are the relative potencies of the analogues as compared with sangivamycin, i.e., IC_{50} sangivamycin/IC_{50} analogue.

the pyrrolopyrimidine nucleosides than RNA synthesis, varies with the cell cycle and has been correlated with chromatin condensation in *Physarum polycephalum* (20–22). In Chinese hamster cells, some histone H1 molecules are modified during interphase to contain one to three serine phosphates and may be correlated with a change in molecular organization (23). Almost all histone H1 molecules are phosphorylated during mitosis at three to six serine and threonine sites and may lead to chromatin condensation (23). Inhibition of this particular postsynthetic modification may not be sufficient by itself to account for cellular cytotoxicity, since disruption of some cell cycle specific events does not necessarily disturb all other cellular functions (24, 25). However, we have examined only a limited class of nuclear proteins, and since regulation of cellular activities by protein phosphorylation is widespread (26), it should be expected that phosphorylation of other nuclear or cytoplasmic constituents may also be inhibited by sangivamycin as suggested by the inhibition of the kinase activities, PK-I and PK-II.

Many protein kinases have been found in nuclei (18, 27) and associated with nucleosomes (28, 29). Apparently, histone H1, HMG 14, and HMG 17 are substrates for different kinase activities, since the inhibition of phosphorylation of histone H1 by sangivamycin was not accompanied by a similar inhibition of HMG 14 and HMG 17. The absence of inhibition of phosphorylation of HMG 14 and HMG 17 in whole cells also serves as an internal control to show that reduced phosphorylation of histone H1 is probably not due to a reduced ATP pool size or reduced ATP specific activity.

Whereas the inhibition of phosphorylation of nuclear proteins in intact cells by the pyrrolopyrimidines is probably due, in part, to the triphosphate, inhibition of the protein kinase activities is a direct competitive effect of the nucleoside, since the enzyme preparations are devoid of nucleoside kinase activity. Thus, sangivamycin, in a manner analogous to cordycepin (2), can inhibit nuclear protein kinase activities. This conclusion is supported by the *in vitro* inhibition of phosphorylation in isolated nuclei. However, the difference in distribution of phosphoproteins in this experiment points out the difficulties in relying solely on such data to derive inferences about the whole cell mechanisms.

In the whole cell assays, toyocamycin was found to inhibit both protein phosphorylation and nRNA synthesis as well as sangivamycin, whereas the amidine and amidoxime analogues were perhaps slightly less effective. Thiosangivamycin was approximately a 50-fold more potent inhibitor of histone H1 phosphorylation than sangivamycin. This difference is in agreement with the relative effects of these drugs on RNA synthesis in L1210 cells (13). These experiments lead to the general conclusion that inhibition of protein phosphorylation may contribute to the cytotoxic effects of these pyrrolopyrimidine analogues.

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