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Žemlička, J., and Chládek, S. (1966), *Collect. Czech. Chem. Commun.* 31, 3775.
Žemlička, J., Chládek, S., Haladová, Z., and Rychlík, I. (1969), *Collect. Czech. Chem. Commun.* 34, 3755.
Žemlička, J., Chládek, S., Ringer, D., and Quiggle, K. (1975), *Biochemistry* 14, 5239.
Žemlička, J., and Murata, M. (1976), *J. Org. Chem.* 41, 3317.
Žemlička, J., Murata, M., and Owens, J. (1977), *ACS Symp. Ser.* (in press).
Žemlička, J., and Owens, J. (1977), *J. Org. Chem.* 42, 517.

Mode of Action of Saframycin A, a Novel Heterocyclic Quinone Antibiotic. Inhibition of RNA Synthesis in Vivo and in Vitro†

Kimiko Ishiguro,* Shigeru Sakiyama, Katsuhiko Takahashi, and Tadashi Arai

ABSTRACT: Saframycin A, an antitumor antibiotic, was shown to preferentially block RNA synthesis in cultured L 1210 cells. The analysis of newly synthesized RNA of the nucleolar or nucleoplasmic fraction of saframycin A treated cells by polyacrylamide gel electrophoresis indicated saframycin A affected the synthesis of both pre-rRNA and heterogeneous nuclear RNA (hnRNA) to the same degree. Furthermore, this antibiotic was characterized as causing a shift of both RNAs toward lower molecular weight regions. Saframycin A alone, on the other hand, failed to affect the template activity of DNA with *Escherichia coli* RNA polymerase in vitro. But the drug showed an inhibitory activity in the presence of dithiothreitol (DTT). The effect was maximized by the pretreatment of

DNA with the drug and DTT and related to the concentration of these two components. The requirement of DTT was slightly replaced by cysteamine, cysteine, and β -mercaptoethanol, but not by NaBH_4 , NAD(P)H, ascorbate, and glutathione. Native conformation of DNA was found to be required for drug action, and the synthetic copolymer, poly(dG)-poly(dC), was more sensitive than poly(dA)-poly(dT). When ^3H -labeled DNA, whose template activity for RNA polymerase was lowered by 90% by the treatment of saframycin A, was subjected to neutral or alkaline sucrose gradient centrifugation, no degradation of DNA was detected. This result suggests that the decreased template activity was not due to strand scission of DNA.

A number of clinically useful antitumor antibiotics interfere with DNA through intercalation, cross-linkage, strand breakage, and other interactions. The inhibition of nucleic acid synthesis might occur preferentially in rapidly proliferating tissues such as tumors. During the course of screening for antitumor antibiotics, new antibiotics, saframycins (A, B, C, D, and E) produced by *Streptomyces lavendulae*, were discovered by the present authors (Arai et al., 1977). The structure of saframycin C (Figure 1) was determined by x-ray crystallography (Tamura et al., unpublished results). The saframycin complex belongs to the group of heterocyclic quinones such as mitomycin C and streptonigrin. However, the structure was characterized by the presence of twin heterocyclic quinones in its skeleton.

Among saframycins, the chemical composition of saframycin A differed from the others in that it possessed an extra nitrogen. Furthermore, saframycin A has been proven to exhibit an extreme cytotoxicity toward cultured cells and to exert an antitumor activity against several experimental tumors including leukemia L 1210 and P 388 and Ehrlich carcinoma, both in ascites and solid forms (Mikami et al., unpublished results). The compound was also active against gram-positive bacteria.

One of our aims is to elucidate the mode of action of saframycins and we chose saframycin A in our experiments because of its high biological activity. In this paper we present the effects of saframycin A on the synthesis of RNA in L 1210 cells and on the transcriptional system in vitro. The unique characteristics of this drug in regard to its requirement of reducing agents to interact with DNA will be presented.

Materials and Methods

Chemicals and Enzymes. Saframycins A and B were prepared in our laboratory, dissolved in methanol at a concentration of 10 mg/mL, and stored at -20°C . None of the effects of the solvent was observed in any assay systems. *Escherichia coli* RNA polymerase (EC 2.7.7.6), poly(dG)-poly(dC), and poly(dA)-poly(dT) were obtained from Boehringer Mannheim Corp. [$5\text{-}^3\text{H}$]Uridine (26.7 Ci/mmol), [$6\text{-}^3\text{H}$]thymidine (13.9 Ci/mmol), a uniformly, ^{14}C -labeled L-amino acid mixture (15 kinds of amino acids, 93–414 mCi/mmol), [$5,6\text{-}^3\text{H}$]UTP (41.4 Ci/mmol), [$5\text{-}^3\text{H}$]CTP (22 Ci/mmol), Aquasol-2, and Protosol were purchased from New England Nuclear, Boston, Mass. Agarose was "Seakem" obtained from Marine Colloids, Inc.

Cells. L 1210 cells were maintained in a suspension culture in Eagle's minimum essential medium supplemented with 10% fetal calf serum as described previously (Ishiguro & Arai, 1976). All experiments were performed with cells in the logarithmic phase of growth.

Macromolecular Synthesis of L 1210 Cells. To determine the effect of saframycin A on the synthesis of RNA, DNA, and protein, cells at a density of 9.45×10^5 cells/mL were treated

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simultaneously with saframycin A and labeled precursors in the following combinations: [^3H]uridine, 0.1 $\mu\text{Ci/mL}$, or [^3H]thymidine, 0.1 $\mu\text{Ci/mL}$, and the ^{14}C -labeled amino acid mixture, 0.2 $\mu\text{Ci/mL}$. At the times indicated, aliquots of 0.1 mL were taken and processed according to the method of Mans & Novelli (1961). The radioactivity was determined by a liquid scintillation counter with toluene-based scintillation fluid.

Cellular Fractionation. Cells (1.5×10^6 cells/mL) in a total volume of 20 mL were treated with saframycin A at a concentration of 0.25 or 0.5 $\mu\text{g/mL}$ for 20 min followed by pulse labeling with [^3H]uridine (1.0 $\mu\text{Ci/mL}$) for 10 min. Then cell suspensions were rapidly poured onto frozen washing medium (phosphate-buffered saline containing 10 mM of unlabeled uridine). Cells were harvested by centrifugation, washed twice, and disrupted according to Penman's procedure (1966), with the exception that Nonidet P-40 was used at a concentration of 0.04% and cell disruption was made with vigorous mixing instead of the use of the homogenizer. The lysate (3 mL) was layered over 10 mL of 50% (w/v) sucrose–2 mM MgCl_2 and centrifuged at 16 000g for 15 min in a swing-out bucket rotor, yielding a nuclear pellet of high purity. The aliquots of 0.1 mL from the supernatant, the cytoplasmic fraction, were mixed with 10 mL of Aquasol-2 for determination of radioactivity. The separation of the nuclear into the nucleolar and nucleoplasmic fractions has been described in detail (Kann & Kohn, 1972).

RNA Extraction. RNA was extracted with a mixture of phenol, *m*-cresol, and 8-hydroxyquinoline twice at 60 $^\circ\text{C}$ for 5 min and once at room temperature by the method of Muramatsu et al. (1970).

Polyacrylamide Gel Electrophoresis. Labeled RNA was analyzed on 2% acrylamide–0.5% agarose gels (6×80 mm) according to Peacock & Dingman (1968). Electrophoresis was performed for 75 min under constant voltage (200 V) which corresponded to 3.3–3.5 mA/gel. After electrophoresis, gels were sliced into 3-mm thicknesses and solubilized with 0.5 mL of Protosol at 55 $^\circ\text{C}$ for 3 h. Radioactivity was then measured with toluene-based scintillator. TMV RNA and rRNA of rat liver served as mobility markers in a parallel gel. The distance of migration was measured with a chromatoscanner (Shimazu, Tokyo, Japan). The molecular weight of RNA was determined using the relation that the mobility is proportional to the log of the molecular weight. *s* values were then calculated with the equation $M = 1550 \times s^{2.1}$ (Spirin, 1961).

In Vitro Transcription. The assay of RNA polymerase was carried out according to the method of Burgess (1971). Enzyme dilution was made with a solution containing 0.01 M Tris-HCl¹ (pH 7.9), 0.01 M MgCl_2 , 0.01 M β -mercaptoethanol, 5×10^{-5} M EDTA, and 50% (v/v) glycerol. The standard assay mixture for polymerization reaction (25 μL) contained: four kinds of ribonucleoside triphosphates (0.08 mM) (UTP carried a tritium label at a radioactive concentration of 20 $\mu\text{Ci/mL}$), 40 mM Tris-HCl (pH 7.9), 0.2 M KCl, 4 mM MgCl_2 , 1 mM MnCl_2 , 20 units/mL RNA polymerase, and 20 $\mu\text{g/mL}$ calf-thymus DNA. After the incubation was carried out for 15 min at 37 $^\circ\text{C}$, 20 μL of aliquot was taken for the measurement of acid-insoluble radioactivity as described previously (Mans & Novelli, 1961).

In experiments involving preincubation of the template or enzyme with saframycin A in the presence of 2 mM DTT, the

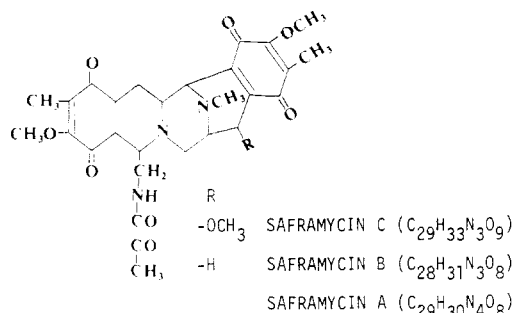


FIGURE 1: Structures and molecular formulas of saframycins C, B, and A.

preincubation mixture was added to the assay mixture of RNA polymerization so as to make the same conditions as in the standard assay with the exception of the inclusion of DTT. The preincubation was carried out for 30 min at 37 $^\circ\text{C}$ unless otherwise stated.

In cases where various DNA templates were preincubated with 2 mM DTT and varying concentrations of the drug, the concentration of DNA was as follows: native or denatured calf-thymus DNA, 50 $\mu\text{g/mL}$; poly(dG)-poly(dC) or poly(dA)-poly(dT), 2.0 A_{260} units/mL; *Bacillus subtilis* DNA, 30 $\mu\text{g/mL}$. When synthetic copolymers were used, the polymerization mixture contained two kinds of corresponding ribonucleoside triphosphates ([^3H]CTP or [^3H]UTP was included at 20 $\mu\text{Ci/mL}$).

Isolation of DNA from *B. subtilis* and Analysis by Sucrose Density Gradient Centrifugation. ^3H -Labeled DNA from *B. subtilis* was prepared according to the method of Marmur (1961) from the cells labeled for 105 min with [^3H]thymidine (0.1 $\mu\text{Ci/mL}$) followed by chase with unlabeled thymidine for 30 min. The final specific radioactivity of DNA was 2438 cpm/ μg . [^3H]DNA (30 $\mu\text{g/mL}$) thus obtained was incubated with saframycin A (50 $\mu\text{g/mL}$) in the presence of 2 mM DTT and 50 mM Tris-HCl (pH 7.9) for 1 h. The DNA samples (50 μL) were layered onto 3.9 mL of an alkaline (0.2 N NaOH, 1.0 M NaCl, and 20 mM EDTA) or neutral (10 mM Tris-HCl (pH 7.4), 1.0 M NaCl, and 1 mM EDTA) sucrose density gradient solution (5–20%). Centrifugation was carried out with the swing-out rotor (6 \times 5 mL) of a MSE 6511/75 centrifuge at 42 000 rpm for 120 min for alkaline or for 75 min for neutral gradient. After centrifugation, ten drops were collected from the bottom of the tubes, mixed with 1 mL of 0.04 N HCl for alkaline or 1 mL of H_2O for neutral gradient, followed by the addition of 10 mL of Aquasol-2, and counted.

Results

Effect on Macromolecular Synthesis. The effect of saframycin A on macromolecular synthesis was studied in an attempt to elucidate the mechanism of action of this drug. The cells were exposed to both drug and radioactive precursors of RNA, DNA, or protein, and radioactivities incorporated into the corresponding fractions were measured at the times indicated. Figure 2 shows a clear gradient of selectivity toward the inhibition of macromolecular synthesis of L 1210 cells. RNA synthesis was most sensitive to the drug and significantly inhibited at a concentration of 0.2 $\mu\text{g/mL}$. At concentrations higher than 2.0 $\mu\text{g/mL}$, synthesis of DNA was also affected, accompanied with the complete inhibition of RNA synthesis. Protein synthesis was least sensitive.

With *Bacillus subtilis*, which is one of the sensitive bacteria to this drug, however, the relation between DNA and RNA synthesis as to the sensitivity against saframycin A was re-

¹ Abbreviations used: hnRNA, heterogeneous nuclear RNA; DTT, dithiothreitol; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; NaBH_4 , sodium borohydride; NADH, nicotinamide adenine dinucleotide, reduced form; NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; UV, ultraviolet.

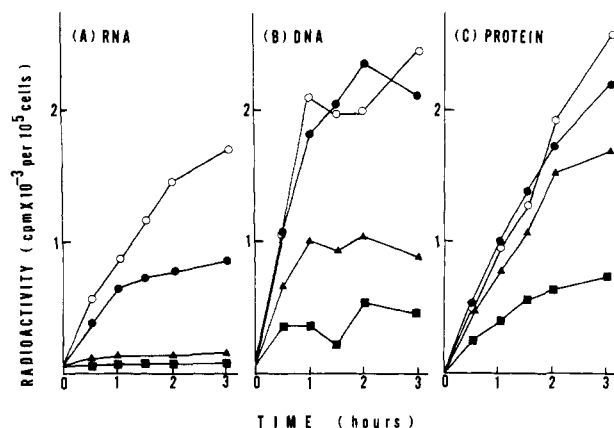


FIGURE 2: Effect of saframycin A on macromolecular synthesis of L 1210 cells. The suspension of L 1210 cells (9.45×10^5 cells/mL) was incubated with the drug at varying concentrations, 0 $\mu\text{g/mL}$ (○), 0.2 $\mu\text{g/mL}$ (●), 2 $\mu\text{g/mL}$ (▲), 20 $\mu\text{g/mL}$ (■), together with [^3H]uridine (A), or [^3H]thymidine (B) and a [^{14}C]labeled amino acid mixture (C). At the times indicated, aliquots of 0.1 mL were taken for determination of radioactivity as described in Materials and Methods.

versed, i.e., the synthesis of DNA was more sensitive than that of RNA. At 30 min after the addition of saframycin A and radioactive precursor, DNA synthesis was inhibited by 71 and 87% at 0.1 and 1.0 $\mu\text{g/mL}$ of the drug, respectively, while the respective values for RNA synthesis were 30 and 78% (data not shown).

In order to examine whether saframycin A inhibits RNA synthesis in a specific manner as exemplified by actinomycin D, it was necessary to perform the direct analysis of hnRNA and pre-rRNA. L 1210 cells were treated with saframycin A followed by 10-min pulse labeling with [^3H]uridine. RNA extracted from nucleolar or nucleoplasmic fraction was then analyzed by polyacrylamide-agarose gel electrophoresis. As shown in Figure 3, pulse-labeled RNA from the nucleolar fraction of untreated cells appeared as a sharp predominant peak at 45 S with a shoulder at 32 S, while that from nucleoplasm showed a broad distribution of radioactivity ranging from 18 to 90 S with a peak at 55 S. Two significant features of the action of saframycin A were observed from these experiments. First, saframycin A did not show any selectivity in the inhibition of nucleolar and nucleoplasmic RNA synthesis. Second, newly synthesized RNA products obtained from cells treated with saframycin A were shortened in chain length as doses were increased.

Radioactivity appearing in various cell fractions in the above experiment is summarized in Table I. Saframycin A at a concentration of 0.25 and 0.50 $\mu\text{g/mL}$ inhibited the total RNA synthesis by 65 and 81%, respectively, whereas the incorporation of [^3H]uridine into the cytoplasmic fraction was barely suppressed, suggesting that saframycin A has no effect on the pool size of uridine or, hence, the transport of uridine.

Effect on *in Vitro* Transcription. The foregoing results which collectively suggest that saframycin A might interact with DNA resulting in the suppression of transcription led us to investigate the effect of this drug on RNA synthesis *in vitro*. This system included calf-thymus DNA, *E. coli* RNA polymerase, and all other prerequisites for transcription. But the result that saframycin A had little effect on this system imposed the dilemma of how the drug could cause severe injury on the transcriptional device *in vivo*. This, however, was solved by the fact that DTT was required for the action of saframycin A *in vitro*. As shown in Figure 4, the introduction of DTT at a concentration of 2 mM into a polymerization reaction mix-

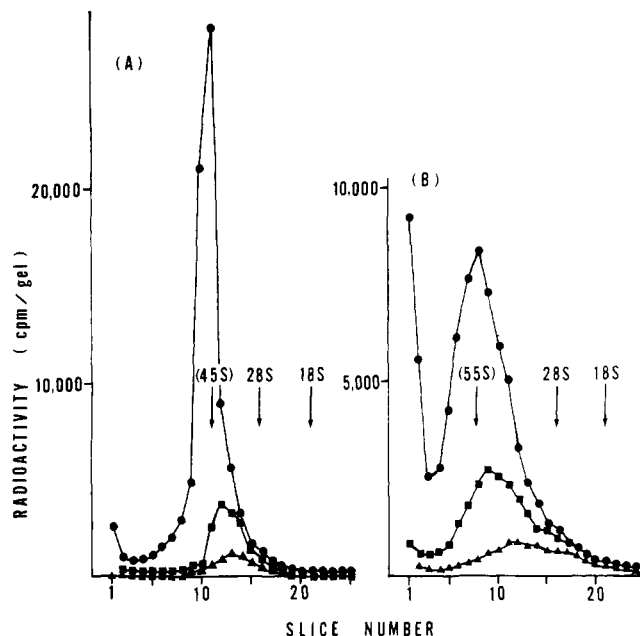


FIGURE 3: Electrophoretic profile of nucleolar and nucleoplasmic RNA from saframycin A treated or untreated L 1210 cells; 3×10^7 cells in a total volume of 20 mL were incubated in the presence or absence of saframycin A for 20 min and labeled with [^3H]uridine for 10 min. RNA extracted from nucleolar (A) or nucleoplasmic (B) fraction was separated by electrophoresis on a 2% polyacrylamide-0.5% agarose gel. Radioactivity in each slice of gel was then determined as described in Materials and Methods. The positions of 18S and 28S rRNA are shown by arrows. Also presented are values calculated as described in Materials and Methods. Saframycin A: 0 $\mu\text{g/mL}$ (●), 0.25 $\mu\text{g/mL}$ (■), or 0.50 $\mu\text{g/mL}$ (▲).

TABLE I: Distribution of [^3H]Uridine in Cytoplasmic Fraction and Nuclear RNA Fractions after Pulse Labeling of Control and Saframycin A Treated L 1210 Cells.^a

Saframycin A ($\mu\text{g/mL}$)	cpm $\times 10^{-3}$			
	In total acid-insoluble fraction	In cytoplasmic fraction	In nucleoplasmic RNA	In nucleolar RNA
0	828 ()	9880 ()	74 ()	73 ()
0.25	288 (65.2)	9510 (3.8)	28 (62.4)	14 (79.9)
0.50	156 (81.2)	8780 (11.1)	12 (83.3)	9 (87.5)

^a Experimental conditions are the same as in the caption of Figure 3. The preparation of subcellular fractions and nuclear RNAs and the measurement of radioactivity were carried out as described in Materials and Methods. Percent inhibition is shown in parentheses.

ture caused approximately 50 and 70% inhibition at 10 and 50 $\mu\text{g/mL}$, respectively, while saframycin A in the absence of DTT gave only a slight degree of inhibition at higher concentrations.

In order to clarify whether the target molecule for saframycin A was template or enzyme, the drug at a concentration of 2 $\mu\text{g/mL}$ was preincubated with DNA or RNA polymerase in the presence of DTT, and thereafter polymerization reaction was initiated. Table II demonstrates that the presence of DNA template in the preincubation mixture caused a greater inhibition (67.5%) than the presence of RNA polymerase (32.1%), suggesting that the drug might affect DNA. The preincubation of the drug with DTT only also gave a lesser extent of inhibition (21.1%). It is noteworthy that the preincubation greatly reduced the concentration of the drug required for efficient inhibition, i.e., 2 $\mu\text{g/mL}$ at which concentration the inhibition

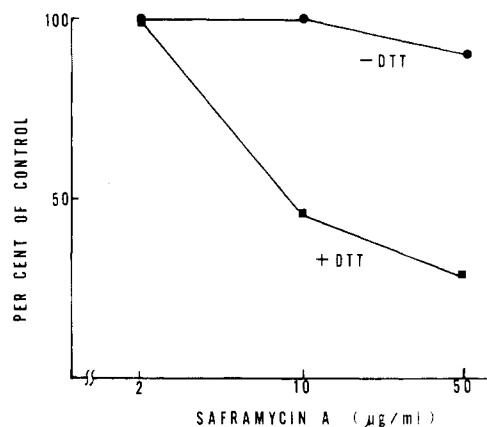


FIGURE 4: Effect of saframycin A on calf-thymus DNA template activity for *E. coli* RNA polymerase in the presence or absence of DTT. A standard assay mixture (25 μ L) containing varying doses of saframycin A in the absence (●) or presence (■) of 2 mM DTT was incubated for 15 min. Aliquots (20 μ L) were withdrawn for counting of radioactivity and processed as described in Materials and Methods.

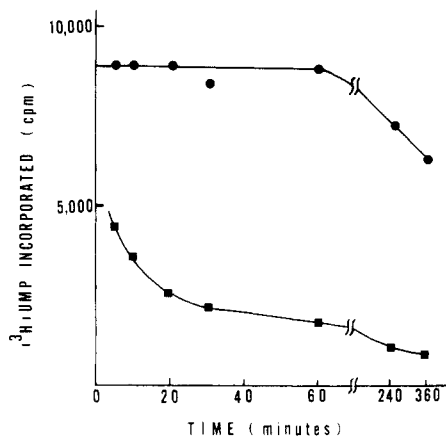


FIGURE 5: Time course of preincubation of saframycin A, DTT, and DNA. Calf-thymus DNA (50 μ g/mL) and DTT (2 mM) in the absence (●) or presence (■) of saframycin A (2 μ g/mL) were preincubated in 50 mM Tris-HCl (pH 7.9) for the times indicated. Thereafter the polymerization reaction was started.

was not observed at all without the preincubation (Figure 4). These results indicate that the simultaneous presence of the drug, DTT, and DNA in the preincubation mixture is necessary for maximum inhibition.

Figure 5 illustrates the time course of preincubation of the drug, DTT, and DNA. After preincubation was carried out for the times indicated, the template activity of DNA was determined. The inhibition was progressively increased up to 20 min and then reached a plateau at about 30 min. Accordingly, preincubation was carried out for 30 min or 1 h in the following experiments in order to obtain efficient inhibition. Further preincubation for more than 4 h retarded the template activity of DNA incubated with DTT alone (control). DTT at a concentration of as high as 2 mM as used in this experiment might induce the degradation of DNA as reported in a previous paper (Bode, 1967).

Figure 6 summarizes the effect of saframycin A on various DNA including artificial DNAs. Saframycin A inhibited the template activity of calf-thymus DNA by 10–90% in a concentration range of 0.017–50 μ g/mL, whereas the inhibition was greatly diminished toward denatured DNA. This finding suggests that saframycin A has a higher affinity toward DNA in native double helical configuration. In order to elucidate

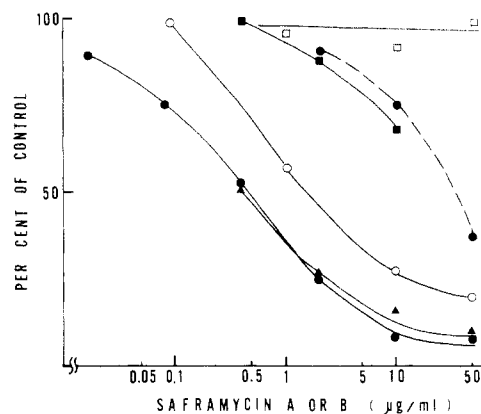


FIGURE 6: Dose dependency of saframycin A or B for the inhibition of various DNA templates. Native (●) or denatured (■) calf-thymus DNA, poly(dG)-poly(dC) (○), poly(dA)-poly(dT) (□), and *B. subtilis* DNA (▲) were preincubated with varying doses of saframycin A in the presence of 2 mM DTT and 50 mM Tris-HCl (pH 7.9). The result obtained from the experiment using saframycin B and native calf-thymus DNA was also included (● - - ●). Preincubation was carried out for 30 min except for 1 h in the case of *B. subtilis* DNA. The values obtained from the experiment without the drug served as control (100%).

TABLE II: Effect of the Preincubation of DNA or RNA Polymerase with Saframycin A on Transcription in Vitro.^a

Saframycin A (2 μ g/mL)	DTT (2 mM)	Addition	[³ H]UMP incorp. (cpm)	% inhibition
—	+	DNA ^b	4770	
+	+	DNA	1550	67.5
—	+	Polymerase ^c	3420	
+	+	Polymerase	2320	32.1
—	+	None	4560	
+	+	None	3590	21.1

^a After the preincubation was carried out in 50 mM Tris-HCl (pH 7.9) for 30 min, 10- μ L portions were added to 15 μ L of the mixture which contained the rest of the components for RNA polymerization. The final condition of each control (without saframycin A) was the same as that of the standard assay except for the inevitable inclusion of DTT. ^{b,c} Calf-thymus DNA or RNA polymerase was added at a concentration of 50 μ g/mL or 50 units/mL, respectively.

whether saframycin A possessed any base specificity, DNA template was replaced by synthetic copolymer, poly(dG)-poly(dC) or poly(dA)-poly(dT). Saframycin A gave the significant inhibition with poly(dG)-poly(dC), while the template activity of poly(dA)-poly(dT) was not affected at all. Therefore, base composition might be an important determinant in interacting with the antibiotic. DNA isolated from *B. subtilis* also showed a behavior similar to native calf-thymus DNA.

Figure 6 also demonstrates the difference between saframycin A and B in inhibiting the template activity of DNA. Saframycin B was about two orders of magnitude less effective than saframycin A. We have also examined the effect of saframycins A and B on cell growth of cultured L 1210 cells. The cells in the exponential phase of growth were exposed to different concentrations of saframycin A or B, and growth was monitored. The growth inhibition by saframycin A was evident at a concentration of as low as 0.002 μ g/mL, while saframycin B was approximately 100–1000 times less effective than saframycin A (data not shown). Thus, a lesser cytotoxicity as well as a weaker antitumor activity in saframycin B might be explained by its low affinity toward DNA. Conversely, the excess nitrogen and carbon in saframycin A might play a significant role in promoting interaction with DNA.

TABLE III: Effect of Various Reducing Agents on the Activation of Saframycin A.^a

Reducing agent	Concn of reducing agent (mM)									
	0.02		0.2		2.0		20.0		200	
	- ^b	+ ^c	-	+	-	+	-	+	-	+
Dithiothreitol	87.9	84.8	97.4	14.1	103	8.9	113	17.4		
Cysteamine					85.7	89.5	94.9	18.2	85.4	14.2
Cysteine					85.6	82.3	80.6	58.1		
β -Mercaptoethanol					103	90.2	99.0	77.4	85.1	28.5
Glutathione					119	109	104	94.4		
Ascorbate	106	118	42.5	58.5	22.0	13.1				
NaBH ₄					59.6	54.8	34.1	39.6		
NADPH					87.7	108				
NADH					104	128				

^a DTT, cysteamine, cysteine, β -mercaptoethanol, glutathione, ascorbate, and NAD(P)H were dissolved in water and adjusted to pH 8 with KOH. Preincubation was carried out for 1 h and results were expressed as percent of control (without reducing agent). ^{b,c} - and + represent the absence and the presence of saframycin A at 10 μ g/mL, respectively, in the preincubation mixture containing DNA (50 μ g/mL) and 50 mM Tris-HCl (pH 7.9).

The reducing agents other than DTT, including cysteamine, cysteine, β -mercaptoethanol, glutathione, ascorbate, NaBH₄, and NAD(P)H were tested and the results are listed in Table III. DTT, when used at concentrations of more than 0.2 mM, exclusively stood out as a potent activating agent. All other sulfhydryl compounds except glutathione were found to possess a potency to activate saframycin A but with a much lesser efficacy compared with DTT. Preincubation with ascorbate or NaBH₄, on the other hand, resulted in a decrease of template activity even without the drug. This decrease is probably due to the strand scission of DNA caused by these agents (Bode, 1967).

The Reduction of Saframycin A in the Presence of DTT. The reduction of saframycin A has been shown by the changes of UV spectra of saframycin A before and after the addition of DTT (Figure 7). The disappearance of a peak of saframycin A at 267 nm together with the appearance of a new peak at 283 nm which represents oxidized DTT (Cleland, 1964) was observed almost immediately after mixing these two agents. The disappearance of UV absorption at 267 nm could be a reflection that the reduction occurs at quinone moieties.

Effect on DNA from *B. subtilis*. In order to investigate whether decreased template activity observed in the RNA polymerase reaction was due to the physicochemical alteration of DNA caused by this drug, ³H-labeled DNA extracted from *B. subtilis* was treated with saframycin A at a concentration of 50 μ g/mL in the presence of DTT for 1 h and analyzed by both neutral and alkaline sucrose density gradient centrifugation. Although the template activity of drug-treated DNA was decreased by almost 90% under these conditions (Figure 7), no fragmentation was detected in either case (data not shown).

Discussion

The data presented in this paper indicate that saframycin A is a potent inhibitor of nucleic acid synthesis of both eukaryotes and prokaryotes. The synthesis of RNA is more sensitive to the drug than that of DNA in L 1210 cells, and vice versa in *B. subtilis*. Although at present there is no way to explain this different effect, some drugs such as adriamycin affect DNA and RNA synthesis in a different degree depending upon the species and the systems tested (Mompalmer et al., 1976; Kitaura et al., 1972).

The selective inhibition of pre-rRNA by actinomycin D or other agents is often simply explained by their high affinity toward G-C pairs. This simple explanation, however, could not

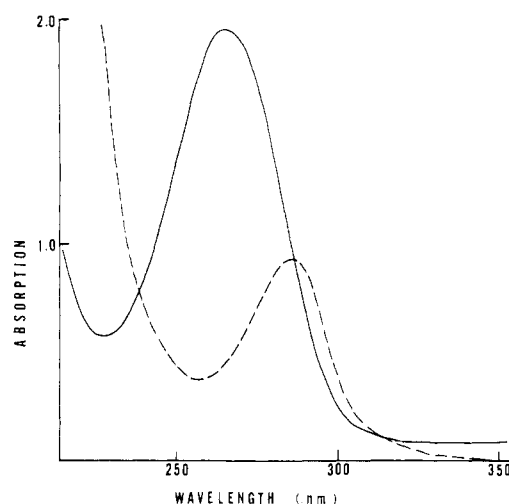


FIGURE 7: Absorption spectra of saframycin A before and after the addition of DTT: saframycin A (50 μ g/mL) with (---) or without (—) DTT (2 mM) in 50 mM Tris-HCl (pH 7.9). The spectra observed at 30 min after mixing the components are shown.

account for the action of saframycin A, since the drug was shown to possess a higher affinity toward G-C pairs in our in vitro experiment, and yet it did not show any selectivity toward rRNA synthesis in vivo. Therefore, it seems likely that not only base composition but also base sequence might be involved in the determinants of selectivity. Furthermore, both species of nuclear RNA extracted from saframycin A treated cells showed a marked reduction in chain length when analyzed by polyacrylamide gel electrophoresis as doses of the drug increased. Possible interpretations as to why this reduction occurred include premature termination or lower rate of elongation of RNA synthesis, breakdown of RNA, or others. But more details of the proposed mechanism require further experimental amplification.

The fact that the inhibition of RNA synthesis in vitro requires sulfhydryl agents indicates that the drug must be transformed to generate an active component. It is expected that the reduction might occur at two quinone moieties in saframycin A. This has been substantiated by the disappearance of the peak of saframycin A at 267 nm after the addition of DTT. There is considerable evidence that quinoid compounds create strand scission of DNA by the production of reduced oxygen species (Cone et al., 1976; Lown & Begleiter,

1976; Lown et al., 1977; Loretzen & Ts'o, 1977). Although it is presumable that a similar mechanism of DNA degradation operates in the interaction of saframycin A with DNA, at present we were unable to detect strand scission of DNA under our experimental conditions. Therefore, the decreased template activity for RNA polymerase caused by saframycin A could possibly be explained by other mechanisms such as intercalation or cross-linkage. In order to clarify whether saframycin A causes strand scission of DNA or not, a more sensitive assay by the use of covalently closed circular DNA should be carried out to detect any amount of strand scission. It has been shown that mitomycin C (Iyer & Szybalski, 1964) and streptonigrin (Cone et al., 1976) were reduced to their hydroquinone derivatives in vitro by NaBH_4 and NADH, respectively. Since neither NaBH_4 nor NADH was found to be an efficient activating agent for saframycin A, the requirement of sulfhydryl agents as reducing agents is a unique characteristic of saframycin A.

The molecular rearrangement of various kinds of drugs in vivo is known to be caused via hepatic biotransformation as shown by benzo[a]pyrene (Gelboin et al., 1972) and cyclophosphamide (Sladek, 1971). In the case of saframycin A, however, such a mechanism can be excluded by its activity toward microorganisms. Therefore, it is plausible that non-specific activation processes common in both eukaryotes and prokaryotes must operate. Our preliminary results showed that cyanide partially reversed the drug-induced inhibition of both RNA synthesis in L 1210 cells and DNA synthesis in *B. subtilis*. This finding suggests that the activation of saframycin A in vivo might be mediated by a process sensitive to cyanide. In this connection, it is interesting to note that cyanide has been shown to enhance the bacteriocidal activity of streptonigrin (White & White, 1968) as well as mitomycin C (White & White, 1965) by promoting intracellular reduction of these agents. Thus, it is assumed that the intracellular reduction of saframycin A and that of streptonigrin or mitomycin C might occur in quite different ways.

Physicochemical changes of DNA caused by saframycin A should be defined in more detail for the understanding of the mechanism of inhibition of nucleic acid synthesis. Studies on this problem are now underway. So far, molecules which require a rearrangement within cells for the action seem to be unique among antibiotics. Thus, the combination of DNA-

interacting ability with this additional property might provide a new approach for cancer chemotherapy.

References

- Arai, T., Takahashi, K., & Kubo, A. (1977) *J. Antibiot.* 30, 1015.
- Bode, V. C. (1967) *J. Mol. Biol.* 26, 125.
- Burgess, R. (1971) *Annu. Rev. Biochem.* 40, 711.
- Cleland, W. W. (1964) *Biochemistry* 3, 480.
- Cone, R., Hasan, S. K., Lown, J. W., & Morgan, A. R. (1976) *Can. J. Biochem.* 54, 219.
- Gelboin, H. V., Kinoshita, N., & Wiebel, F. J. (1972) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* 31, 1298.
- Ishiguro, K., & Arai, T. (1976) *Antimicrob. Agents Chemother.* 9, 893.
- Iyer, V. N., & Szybalski, W. (1964) *Science* 145, 55.
- Kann, H. E., Jr., & Kohn, K. W. (1972) *J. Cell. Physiol.* 79, 331.
- Kitaura, K., Imai, R., Ishihara, Y., Yanai, H., & Takahira, H. (1972) *J. Antibiot.* 25, 509.
- Loretzen, R. J., & Ts'o, P. O. P. (1977) *Biochemistry* 16, 1467.
- Lown, J. W., & Begleiter, A. (1976) *Can. J. Biochem.* 54, 110.
- Lown, J. W., Sim, S.-K., Majumdar, K. C., & Chang, R.-Y. (1977) *Biochem. Biophys. Res. Commun.* 76, 705.
- Mans, R. J., & Novelli, G. D. (1961) *Arch. Biochem. Biophys.* 94, 48.
- Marmur, J. (1961) *J. Mol. Biol.* 3, 208.
- Momparler, R. L., Karon, M., Siegel, S. E., & Avila, F. (1976) *Cancer Res.* 36, 2891.
- Muramatsu, M., Shimada, N., & Higashinakagawa, T. (1970) *J. Mol. Biol.* 53, 91.
- Peacock, A. C., & Dingman, C. W. (1968) *Biochemistry* 7, 668.
- Penman, S. (1966) *J. Mol. Biol.* 17, 117.
- Sladek, N. E. (1971) *Cancer Res.* 32, 901.
- Spirin, A. S. (1961) *Biokhimiya* 26, 511.
- White, J. R., & White, H. L. (1965) *Antimicrob. Agents Chemother.* 1964, 495.
- White, H. L., & White, J. R. (1968) *Mol. Pharmacol.* 4, 549.