Regulation of Ribosomal RNA Synthesis in Mammalian Cells: Effect of Toyocamycin†

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ABSTRACT: The present study shows that the antitumor agent toyocamycin (4-amino-5-cyano-7β-D-ribofuranosylpyrrolo(2-3*d*)pyrimidine) affects rRNA transcription in Ehrlich ascites cells. This action of the antibiotic is dependent on the amino acid composition of the cell culture medium. In cells incubated in a medium rich in amino acids, the high transcription rate of rRNA is lowered by the addition of 2×10^{-6} M toyocamycin, while in amino acid starved cells the decreased level of rRNA synthesis remains unaffected. Processing of the 45S rRNA precursor is markedly inhibited by toyocamycin in cells incubated in either medium, indicating that the uptake of the drug is unimpaired by amino acid starvation. Toyocamycin does not affect RNA polymerase I (RNA nucleotidyltransferase EC 2.7.7.6) activity when added to in vitro assay systems derived from cells grown in complete or in amino acid deficient media. The drug prevents the activation of rRNA synthesis following the refeeding of amino acid starved cells without affecting the stimulation of protein synthesis.

When mammalian cells are exposed to media with different amino acid concentration there is a rapid change in the rate of ribosome synthesis (Shields and Korner, 1970) as well as in the RNA polymerase I[1] activity in isolated nuclei (Franze-Fernández and Pogo, 1971; Grummt et al., 1976). The mechanism underlying this regulation is as yet unknown. The fact that protein synthesis is required to activate nuclear polymerase I by amino acids (Franze-Fernández and Fontanive-Sanguesa, 1973) and the demonstration that rRNA transcription in eukaryotes is very sensitive to inhibition of protein synthesis (Willems et al., 1969; Muramatsu et al., 1970; Benecke et al., 1973; Franze-Fernández and Fontanive-Sanguesa, 1973; Gross and Pogo, 1976; Penman et al., 1976) suggest that protein synthesis is involved in this process. However, other factors may also be operating, as indicated by the observation that the amino acid requirement for RNA polymerase I activation differed from that for stimulus to protein synthesis (Franze-Fernández and Fontanive-Sanguesa, 1973).

With a view to determine whether the mechanism controlling the rate of rRNA transcription by amino acids can be affected without an alteration in protein synthesis, we have been investigating the action of several antibiotics. We report herein the results obtained with toyocamycin. This adenosine analogue is an antitumor agent known to inhibit rRNA maturation (Tavitian et al., 1968; Weiss and Pitot, 1974). We have found that toyocamycin (2×10^{-6} M) prevents the activation of rRNA synthesis upon refeeding amino acid starved Ehrlich ascites cells, without interfering with the stimulation of protein synthesis. The data seem to indicate that toyocamycin affects the mechanism regulating the rate of rRNA transcription.

Materials and Methods

(*a*) *Obtention and Culture of Ehrlich Ascites Cells.* The cells used were grown for 5–7 days in the abdominal cavity of mice. The preparations of the cells and of the incubation media with and without amino acids were carried out as described previously (Franze-Fernández and Pogo, 1971). Cells were incubated at a concentration of 2×10^6/mL medium. For all experiments, the cells were preincubated 1 h in an amino acid deprived medium and then collected and suspended in the appropriate medium and further incubated for the times indicated. In the experiments in which the cells were transferred to a medium containing toyocamycin, they were exposed to the antibiotic for the last 10 min of the preincubation period.

(*b*) *Nuclear Isolation.* Nuclei were purified as previously indicated (Franze-Fernández and Fontanive-Sanguesa, 1973).

(*c*) *DNA-Dependent RNA Polymerase I Assay.* RNA polymerase I in isolated nuclei was assayed by measurement of the incorporation of [5-^{3}H]UTP[2] (50–60 Ci/mol) into RNA as described (Franze-Fernández and Fontanive-Sanguesa, 1973), with the modification that the final volume of the assay mixture was 0.13 mL and contained 40–50 μg of DNA of the nuclear suspension. All the assays were performed in the presence of 3 μg/mL of α-amanitin. No further inhibition of the activity was found when the assays were performed with 150 μg/mL of α-amanitin indicating that, in our nuclei preparation, the α-amanitin insensitive activity corresponds to RNA polymerase I (Weil and Blatti, 1976). When 400 μg/mL of heparin was added to the assay mixture the polymerase activity was not affected. Since heparin is known to inhibit RNA chain initiation (Udvardy and Seifart, 1976), this showed that the activity measured in isolated nuclei corresponds to the enzyme already bound to the DNA template.

The assay of isolated RNA polymerase I was performed as described previously (Cereghini and Franze-Fernández, 1974), with the difference that native calf thymus DNA (type I from Sigma) was used and that the reaction was incubated 15 min.

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[1] Nuclear RNA polymerase I is responsible for the synthesis in vivo of precursor rRNA (Reeder and Roeder, 1972).

[2] Abbreviations used: UTP, uridine triphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; DTE, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl.

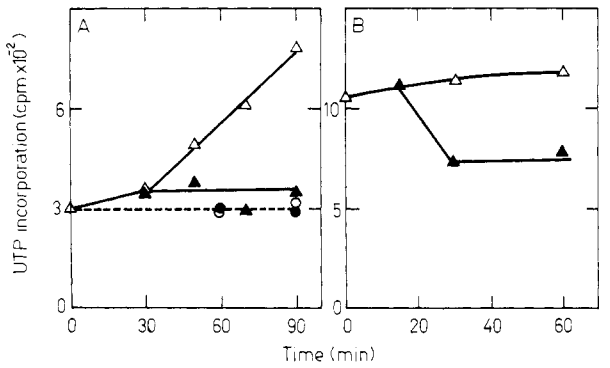

FIGURE 1: Effect of toyocamycin on nuclear RNA polymerase activity in cells cultured in complete and in amino acid free media. (A) Cells precultured in an amino acid deprived medium were spundown and further incubated in: a complete medium (—△—); a complete medium plus 2 μM toyocamycin (—▲—); an amino acid free medium (- -○- -); an amino acid free medium with 2 μM toyocamycin (- -●- -). At the times indicated, nuclei were isolated and the RNA polymerase activity was determined. (B) Cells were precultured in a complete medium for 80 min. At zero time, toyocamycin was added up to a final concentration of 2 μM to half of the culture and the incubation was continued. Aliquots of the incubate were taken at the times indicated from the control (—△—) and the toyocamycin-treated culture (—▲—); the nuclei were isolated and the RNA polymerase activity was determined.

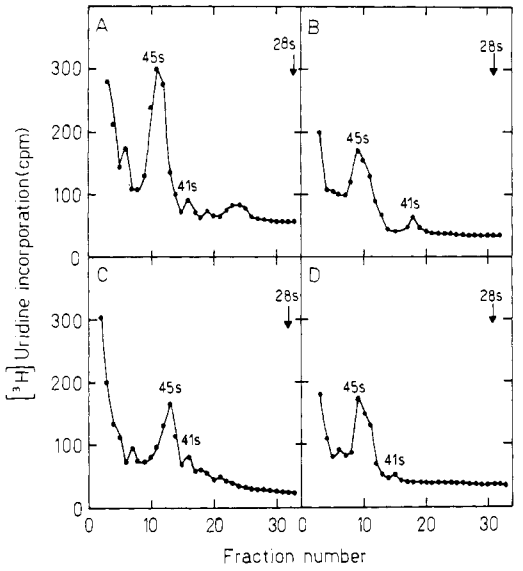

FIGURE 2: Effect of toyocamycin on the incorporation of [^{3}H]uridine in control and in amino acid deprived cells. Ehrlich ascites cells preincubated in an amino acid deprived medium were transferred to: (A) a complete medium; (B) a complete medium containing 2 μM toyocamycin; (C) an amino acid deprived medium; (D) an amino acid deprived medium plus 2 μM toyocamycin. The incubation was continued for 1 h, where upon the cells were pulse labeled with [^{3}H]uridine (10 μCi/mL) for 10 min. Total cell RNA was purified and an amount corresponding to about 3.5×10^5 cells was analyzed by electrophoresis in 2.4% acrylamide gels at 6 mA/gel for 5.5 h.

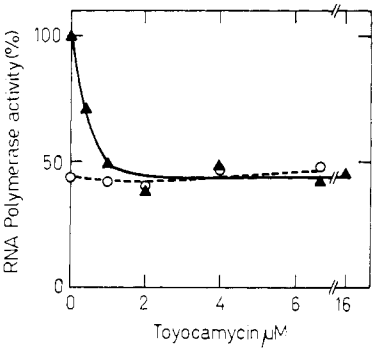

FIGURE 3: Effect of addition of different concentrations of toyocamycin on nuclear RNA polymerase activity. Cells cultured in a complete (—▲—) or in an amino acid deprived medium (- -○- -) were exposed to different concentrations of toyocamycin for 1 h. At this time, the transcriptional activity in isolated nuclei was determined.

All assays were carried out in the presence of 4 μg/mL of α-amanitin.

(*d*) *Purification of RNA Polymerase I.* The RNA polymerases were solubilized from nuclei and precipitated with ammonium sulfate according to the procedure previously described (Cereghini and Franze-Fernández, 1974). The ammonium sulfate precipitate from about 1 g of cells was resuspended in 1-2 mL of buffer R (0.05 M Tris-HCl (pH 7.90), 5 mM $MgCl_2$, 0.5 mM DTE, 0.1 mM EDTA, 0.3 mg/mL each of Dextrans no. T 70, T 150, T 250, 30% glycerol). This solution (fraction 2) was diluted with buffer R to bring the ammonium sulfate concentration to 0.05 M and chromatographed on a DEAE-Sephadex A-25 (Pharmacia) column as indicated (Cereghini and Franze-Fernández, 1974).

(*e*) *Labeling and Analysis of the RNA.* Cells were labeled with [5-^{3}H]uridine (20 Ci/mmol) for the times indicated and the incorporation was halted by pouring the cells into frozen Dulbecco's medium. The cells were collected by centrifugation and dissolved in a buffer containing: 0.1 M NaCl, 0.1 M Tris-HCl (pH 7.20), 0.001 M EDTA, and 0.5% sodium dodecyl sulfate. At this stage, a tracer amount of ^{14}C-labeled rRNA was added and total cell RNA was purified and analyzed by electrophoresis in acrylamide gels as previously described. The radioactivity in the rRNA precursor was estimated graphically (Franze-Fernández and Fontanive-Sanguesa, 1975).

(*f*) *Measurement of the Incorporation of Radioactive Amino Acids into Protein.* Cells labeled with [4-5-^{3}H]leucine (10 Ci/mol) were poured into 10 mL of cold Dulbecco's saline containing 0.4 μmol of cold leucine and sedimented by centrifugation. The pellet was dissolved in 2 mL of 1 M KOH, allowed to stand for 10 min at room temperature, and precipitated with 1.5 mL of 50% trichloroacetic acid (Hogan and Korner, 1968). The precipitate was collected by centrifugation and washed three times with 7 mL of 5% trichloroacetic acid. The final precipitate was dissolved and the radioactivity counted as described elsewhere (Franze-Fernández and Pogo, 1971).

Results

Effect of Toyocamycin on rRNA Transcription. It has been previously demonstrated that soon after Ehrlich ascites cells are exposed to a medium rich in amino acids there is an increase in the nuclear RNA polymerase I activity (Franze-Fernández and Pogo, 1971).

As illustrated in Figure 1A, toyocamycin (2×10^{-6} M) prevents the activation of rRNA transcription following the refeeding of amino acid starved cells. The transcriptive capacity in nuclei from cells cultured in an amino acid deprived medium is not affected by the drug. In Figure 1B, it is shown that, after addition of toyocamycin to cells previously activated by amino acids, there is a rapid decline in the nuclear form I enzyme activity.

The effect of the analogue on the rate of rRNA synthesis in whole Ehrlich ascites cells was also examined. For that purpose, control and toyocamycin treated cells were labeled with a short pulse of [^{3}H]uridine, the RNAs were purified and

TABLE I: Nuclear RNA Polymerase I Activity and Rate of rRNA Precursor Synthesis in Control and in Toyocamycin-Treated Cells.

Culture medium[a]	Experiment 1		Experiment 2	
	[^{3}H]Uridine incorp. into RNA[b] (%)	RNA polymerase I act.[c] (%)	[^{3}H] Uridine incorp. into RNA[b] (%)	RNA polymerase I act.[c] (%)
With amino acids	100	100	100	100
With amino acids + toyocamycin (2×10^{-6} M)	59	58	57	47
Without amino acids	34	50	41	50
Without amino acids + toyocamycin (2×10^{-6} M)	45	49	39	49

[a] Cells preincubated in an amino acid deprived medium were transferred to the media indicated and incubation was continued for 1 h. [b] An aliquot of each culture was then removed and pulse-labeled with [^{3}H]uridine (5 μCi/mL for 20 min in experiment 1; 10 μCi/mL for 10 min in experiment 2). Total cell RNA was purified and analyzed by acrylamide gel electrophoresis as indicated in Figure 2 and the radioactivity in the rRNA precursor peaks estimated. [c] Nuclei were isolated from the remaining culture and the RNA polymerase activity was determined. The values obtained in cells incubated in a complete medium were considered to be 100%.

TABLE II: Effect of Addition of Toyocamycin to Isolated Nuclei.

Culture medium[a]	RNA polymerase I act.[b] (cpm)			
		Toyocamycin		
		2×10^{-6} M	1×10^{-5} M	4×10^{-5} M
With amino acids	1110	1160	1010	1100
With amino acids + toyocamycin (2×10^{-6} M)	460	460	518	470
Without amino acids	270	300	280	260
Without amino acids + toyocamycin (2×10^{-6} M)	300	290	250	260

[a] Cells precultured in an amino acid deprived medium were transferred to the media indicated and incubation was continued for 80 min. [b] Nuclei were then isolated and the RNA polymerase I activity was assayed without or in the presence of the indicated concentrations of toyocamycin.

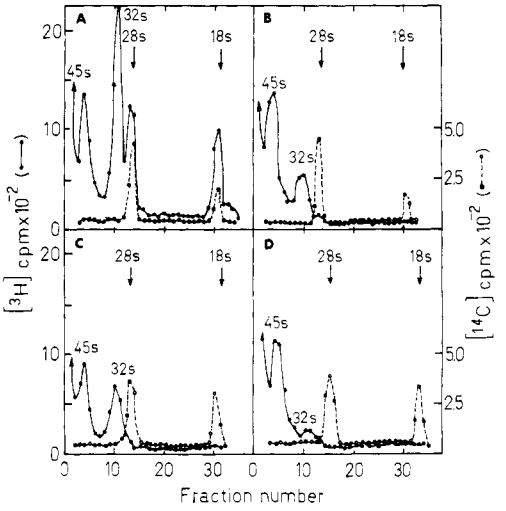

FIGURE 4: Effect of toyocamycin on rRNA maturation. Cells precultured in an amino acid free medium were transferred to: (A) a complete medium; (B) a complete medium plus 2 μM toyocamycin; (C) an amino acid deprived medium; (D) an amino acid deprived medium with 2 μM toyocamycin. All media contained 10 μCi/mL of [^{3}H]uridine. After 2 h of labeling, total cell RNA samples were prepared and the RNA from about 2.5×10^5 cells of each sample was analyzed by electrophoresis in 2.7% acrylamide gels at 6 mA/gel for 4.5 h. (—●—) [^{3}H]Uridine incorporation; (- -○- -) ^{14}C-labeled rRNA marker.

analyzed by polyacrylamide gel electrophoresis, and the amount of label incorporated into the rRNA precursor was estimated. A typical analysis of the labeled RNAs is illustrated in Figure 2, and the results of two independent experiments are summarized in Table I. It was found that in amino acid deprived cells rRNA transcription continues, although at a reduced rate compared with control cells. In agreement with the observations on isolated nuclei, toyocamycin lowers rRNA synthesis in cells incubated in a complete medium but does not affect rRNA transcription in amino acid starved cells. The results in Table I show that there is a close correlation between the rate of rRNA synthesis in whole cells and the corresponding data of nuclear RNA polymerase I activity indicating that isolated nuclei are a suitable system for studying the effect of toyocamycin on rRNA transcription.

The experiment illustrated in Figure 3 shows the concentration dependence of the inhibition of nuclear RNA polymerase I activity by toyocamycin. In the cells incubated in a complete medium the maximal inhibition is attained at a concentration of 2×10^{-6} M toyocamycin. Transcription in nuclei from amino acid starved cells was not affected at any of the concentrations of toyocamycin tested.

Unlike the in vivo effect, toyocamycin does not inhibit RNA polymerase I activity when added to in vitro assay systems derived from cells grown in media either with or without amino acids. This was found both in the isolated nuclei system (Table II) and also when the extracted enzymes were assayed with an exogenous DNA template (results not shown). Similar results were obtained with toyocamycin monophosphate (results not shown).

Effect of Toyocamycin on rRNA Processing. The failure of toyocamycin to affect rRNA synthesis in cells incubated in a medium without amino acids might be the consequence of an altered uptake of the antibiotic. If this were the case, toyocamycin should not inhibit the maturation of the rRNA precursor (Tavitian et al., 1968), in amino acid starved cells. We therefore investigated this effect of the drug on cells incubated in complete and in amino acid deficient media. The results in Figure 4 show that toyocamycin, at a concentration of 2×10^{-6} M, severely inhibits the processing of the 45S into 32S rRNA precursor in cells incubated in either medium indicating that the uptake of the drug is not affected by amino acid starvation.

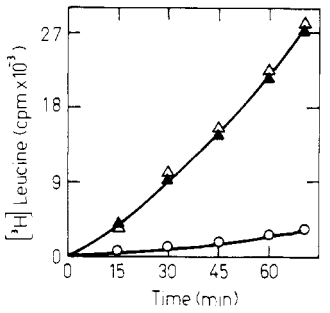

FIGURE 5: Effect of toyocamycin on the incorporation of [^{3}H]leucine into proteins. Cells preincubated in amino acid-deprived medium were spun-down and further incubated in: a complete medium (—△—); a complete medium plus 2 μM toyocamycin (—▲—); an amino acid free medium (—○—). All media contained 1 μCi/mL of [^{3}H]leucine. At the times indicated, aliquots of about 1.5×10^6 cells were taken and poured on cold Dulbecco's saline and processed as described in Materials and Methods.

TABLE III: Effect of Toyocamycin and Amino Acid Deprivation on the Yields of Soluble RNA Polymerase I.

Culture medium[a]	RNA polymerase I act.[b] (pmol/g cells)	
	Fraction 2	DEAE-Sephadex chromatography
With amino acids	2600	2650
With amino acids + toyocamycin (2×10^{-6} M)	2350	2340
Without amino acids		2560

[a] Ehrlich ascites cells preincubated in an amino acid free medium were transferred to the media indicated and further incubated 75 min. [b] Nuclei were then isolated and the RNA polymerases were solubilized and chromatographed on DEAE-Sephadex A-25, as indicated in Materials and Methods.

Effect of Toyocamycin on Protein Synthesis. Studies from several laboratories have established that in mammalian cells there is a linkage between the rate of rRNA synthesis and that of protein synthesis (Willems et al., 1969; Muramatsu et al., 1970; Franze-Fernández and Fontanive-Sanguesa, 1973; Penman et al., 1976). To ascertain if toyocamycin affects rRNA transcription as a result of the inhibition of protein synthesis, we compared the incorporation of [^{3}H]leucine into proteins in cells incubated in a complete medium with and without toyocamycin. The results in Figure 5 show that protein synthesis proceeds normally in toyocamycin-treated cells.

Effect of Toyocamycin on the Activity of Isolated RNA Polymerase I. It has been pointed out that the RNA polymerases extracted from nuclei of Ehrlich ascites cells are resolved in two peaks of activity by DEAE-Sephadex chromatography. The first peak elutes at 0.06–0.10 M ammonium sulfate, and the second at about 0.25 M of the salt. With respect to this and other properties, these peaks correspond to the RNA polymerases I and II from other eukaryotic organisms (Cereghini and Franze-Fernández, 1974). The results in Table III indicate that the amount of RNA polymerase I activity detected with exogenous DNA template in crude nuclear extracts or after DEAE-Sephadex chromatography is not modified by amino acid starvation or treatment with toyocamycin. Thus, only the activity of the enzyme bound to the DNA template is affected. This is consistent with previous results that demonstrated that the level of soluble RNA polymerase I does not change by inhibition of protein synthesis (Cereghini and Franze-Fernández, 1974).

Discussion

To our knowledge, this report presents the first demonstration that toyocamycin affects rRNA transcription and that this action of the drug is dependent on the nutritional state of the cells. In cells incubated in a medium rich in amino acids, 2×10^{-6} M toyocamycin lowers the rate of rRNA transcription; the basal level of rRNA synthesis in amino acid starved cells is unaffected even at higher concentrations of toyocamycin, notwithstanding the fact that the uptake of the analogue is unimpaired by amino acid deprivation. Addition of toyocamycin to cells grown in a complete medium affects both the rate of the [^{3}H]uridine incorporated into the 45S rRNA precursor, and also the activity of the RNA polymerase I in the isolated nuclei; the latter assay reflects the activity of enzyme I molecules which had been initiated in vivo, since in our nuclei preparation no initiation of RNA chains occurs (see Materials and Methods). Unlike the in vivo effect, toyocamycin does not inhibit polymerase I activity when directly added to in vitro assay systems derived from cells grown in media either with or without amino acids. These results lend support to the suggestion that toyocamycin has no direct effect on transcription; the drug, instead, may alter the mechanism regulating the rate of rRNA synthesis.

It has been shown that toyocamycin does not inhibit protein synthesis. Thus, other alternatives must be considered to explain the observed effect on transcription.

It has been demonstrated in yeast that mutations in nine distinct genes that regulate ribosome biosynthesis affect both transcription and maturation of rRNA (Warner and Udem, 1972). In mammalian cells, inhibition of protein synthesis also alters both processes (Craig, 1974). These findings suggest that transcription and processing of rRNA in eukaryotes are stringently regulated. Therefore, one possibility to be considered is that the interruption of the processing of the 45S rRNA by toyocamycin acts as a signal for the inhibition of rRNA transcription. Our results indicate that, if there is an association between these steps of the ribosome biosynthesis, it is not absolute. In amino acid starved cells, where maturation of the rRNA precursor has already declined to about one-third with respect to control cells (see Figure 4), further inhibition of the rRNA processing by toyocamycin does not affect transcription.

Since in bacteria the cellular mediators for the stringent response to amino acid starvation are modified nucleotides (Cashel and Gallant, 1969; Haseltine et al., 1972), another possibility is that toyocamycin, being an adenosine analogue, interferes with the action of some unknown nucleotide mediating the response to amino acid availability in animal cells. Toyocamycin, for instance, might mimic the effect of a factor involved in the negative control of rRNA synthesis; the fact that phenotypic relaxation has been observed in yeast (De Kloet, 1966; Foury and Goffeau, 1973; Gross and Pogo, 1974, 1976) suggests the existence of a repressor-like component in eukaryotes. Another alternative is that toyocamycin antagonizes the effect or inhibits some enzymatic reaction that would otherwise lead to the formation of an activator of rRNA transcription. The recent finding that the level of a highly phosphorylated adenosine dinucleotide is correlated with the proliferative activity in different mammalian cells (Rapaport and Zamecnik, 1976) points to the existence of such positive effectors. It is noteworthy that another adenosine analogue, tubercidin, affects rRNA transcription in a similar manner to

toyocamycin (S. Iapalucci-Espinoza and M. T. Franze-Fernández, unpublished results).

That the amount of RNA polymerase I is not the rate-limiting factor in rRNA synthesis was shown when the enzyme was isolated from cells cultured in an amino acid deprived medium or in complete medium with or without toyocamycin. This is consistent with similar findings obtained with RNA polymerase I extracted from Ehrlich ascites and HeLa cells cultured with inhibitors of protein synthesis (Cereghini and Franze-Fernández, 1974; Chesterton et al., 1975). This suggests that the rapid changes in the rate of rRNA transcription that occur by the action of these agents presumably involves modulation of the activity of preexisting RNA polymerase I molecules. This modulation could, among other possibilities, be the consequence of a rapid turning over polypeptide factor required for catalysis as suggested by Lampert and Feigelson (1974), Cereghini and Franze-Fernández (1974), Chesterton et al. (1975), and Gross and Pogo (1976).

Acknowledgments

We wish to thank Professor Th. Wieland (Max Plank Institute, Heidelberg, Germany) for his generous gift of α-amanitin and also Dr. G. Acs for his great kindness in furnishing us with toyocamycin and its monophosphate.

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