MECHANISM OF ACTION OF ACETYL KIDAMYCIN

II. INHIBITION OF RNA SYNTHESIS IN HeLa CELLS

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The effects of acetyl kidamycin on RNA synthesis in HeLa cells were investigated. Acetyl kidamycin inhibited the synthesis of 45S pre-rRNA, heterogeneous nuclear RNA(HnRNA) and small molecular weight RNAs, though not to the same degree. The processing of 45S pre-rRNA into 18S and 28S rRNA and that of HnRNA into mRNA were not affected.

We had previously reported that acetyl kidamycin, an anthracycline antibiotic having antitumor activity[1], binds strongly to DNA and, in consequence, increases the melting temperature of DNA and decreases its buoyant density. Furthermore it brings about a single strand scission of double-stranded DNA.[2]

Subsequent studies have been directed towards further elucidation of the mechanism by which RNA synthesis is inhibited by the antibiotic.

This report will show the effect of acetyl kidamycin on RNA synthesis in HeLa cells.

Materials and Methods

HeLa cells were grown in suspension culture to a density of $2\sim4\times10^5$ cells/ml in EAGLE's medium[3] supplemented with 10% calf serum (Flow Laboratories, U.S.A.). Prior to labeling, cells were concentrated to 2×10^6 cells/ml.

Nuclear RNA was labeled with a 10-minute pulse of [5-^{3}H] uridine (26.6 mCi/mM, New England Nuclear). For the study of 45S pre-rRNA processing, a high level of actinomycin D (5μg/ml) was added to the culture after the 10-minute pulse in order to stop the further synthesis of pre-rRNA, then rRNA was permitted to mature in the cytoplasm for 2 more hours. All isotope incorporations were terminated by pouring the cultures over frozen EARLE's balanced salt solution. Cells were harvested by centrifugation, washed twice with cold EARLE's saline and then resuspended in cold hypotonic buffer RSB (reticulocyte standard buffer; 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris, pH 7.4) prior to breaking with detergent (NP 40). Nuclear and cytoplasmic fractions were separated by centrifugation at $800\times g$ for 2 minutes. The resulting nuclear pellet was washed and then resuspended in the same buffer RSB. The RNA was extracted by the hot phenol-chloroform method[4] and analyzed by sucrose density-gradient centrifugation with sucrose-SDS[5] (sodium dodecyl sulfate).

Each fraction was monitored for absorbance (254 nm), precipitated with cold 10% trichloroacetic acid, filtered onto Millipore filters, rinsed three times with cold 5% trichloroacetic acid, dried and counted in a toluene-based scintillator.

For labeling of cytoplasmic mRNA, cells were preincubated with a low level of actinomycin D (0.04 μg/ml) for 30 minutes then labeled with [5-^{3}H]uridine for 20 minutes. The labeling was terminated by the addition of high level of actinomycin D (5 μg/ml) and then mRNA was allowed to pass into the cytoplasmic fraction for 1 hour more. The disruption of cells, extraction and analysis of RNA were done by the same method as described above.

Poly(A)-containing RNA, which was isolated from nuclei or cytoplasm, was bound to poly(U)-Sepharose (Pharmacia Fine Chemicals, Sweden) at room temperature in binding buffer (0.1 M sodium phosphate, 0.12 M NaCl, 0.1 mM EDTA, 10MM-Tris, pH 7.3). Poly(U)-Sepharose column (0.2 ml)

and then washed with the same buffer (lacking the sodium phosphate) in order to reduce non-specific binding. Poly(A)-containing RNA was eluted from the Sepharose column with 50% formamide at room temperature.[6,7]

Results

The effect of acetyl kidamycin on the labeling of ribosomal precursor RNA (45S RNA) and HnRNA is shown in Fig. 1. In this 10-minute pulse, there is a significant inhibition of 45S pre-rRNA synthesis. This inhibition reached approximately 60% in the presence of 1.0 μg/ml of the drug. It has been shown that a high level of actinomycin D (5 μg/ml) does not inhibit the processing of nucleolar RNA, but inhibits new synthesis of all RNA.[8] However, low levels of actinomycin D, at concentrations of 0.01 to 0.05 μg/ml, selectively inhibit the transcription of 45S pre-rRNA alone.[9,10,11]

Fig. 1. Inhibition of nuclear RNA synthesis by acetyl kidamycin.

HeLa cells were labeled with a 10-minute pulse of [^{3}H]uridine (10 μCi/ml). In the drug-treated cells, acetyl kidamycin (1.0 μg/ml) was added 30 minutes prior to labeling. RNA was isolated from nuclei and extracted as in Materials and Methods. Velocity sedimentation in 15~30 % sodium dodecyl sulfate-sucrose gradients was carried out in a Spinco SW 50 rotor at 25,000 rev/min for 10 hours at 20°C. 28S RNA from HeLa cells was used as a marker as indicated by arrows.

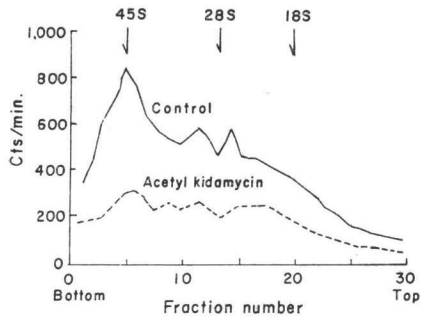


Fig. 3. Inhibition of mRNA synthesis by acetyl kidamycin.

In the presence of actinomycin D (0.04 μg/ml), HeLa cells were incubated for 30 minutes with or without acetyl kidamycin (0.1 μg/ml), and then labeled with [^{3}H]uridine (10 μCi/ml) for 2 hours. Centrifugation as in Fig. 1.

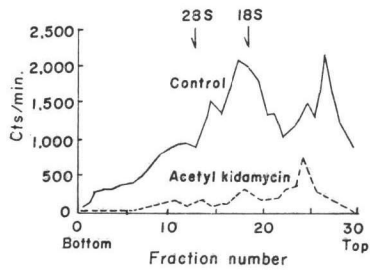
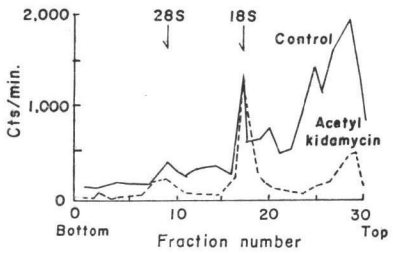


Fig. 2. Effect of acetyl kidamycin on the processing of pre-ribosomal RNA.

After a 10-minute pulse with [^{3}H]uridine (10 μCi/ml), actinomycin D (5 μg/ml) was added to HeLa cells and the cells were incubated for an additional 2 hours. In the drug-treated cells, acetyl kidamycin (10 μg/ml) was added at the same time as actinomycin D addition. Centrifugation as in Fig. 1.



By taking advantage of these facts, the following experiments were done. As shown in Fig. 2, there is no apparent inhibition of the processing of 45S pre-rRNA into 28S and 18S RNA even at 10 μg/ml of acetyl kidamycin, though the labeling of small molecular weight RNA in the cytoplasm was markedly inhibited. Previous work indicated that the first precursor to ribosomal RNA synthesized is 45S RNA,[12] and that this is later cleaved into 32S and 18S RNA.[8,13] The 18S RNA is rapidly transferred to the cytoplasm. The 32S RNA remains in the nucleolus and is transformed into nuclear 28S RNA; at later times cytoplasmic 28S RNA begins to appear. On this basis, the results in Fig. 2 are consistent in that the labeling ratio of 28S RNA

Table 1. Effect of acetyl kidamycin on the synthesis of poly(A)-containing RNA in nuclei

Acetyl kidamycin (μg/ml)	Poly(A)-containing RNA in nuclei (cpm)	% Inhibition
0	4201.5	0
0.05	306.7	82.7
0.1	146.7	96.5
1.0	62.1	98.5

HeLa cells (2.5×10^6) were incubated with actinomycin D (0.04 μg/ml) for 30 minutes in the presence or absence of acetyl kidamycin at the indicated concentrations. Then RNA was labeled with [5-^{3}H]uridine (10 μCi/ml) for 10 minutes, and the labeling was terminated by pouring the cultures over frozen EARLE's balanced salt solution.

The isolation of RNA and the purification of poly(A)-containing RNA were carried out as in Materials and Methods. Portions of poly(A)-containing RNA were collected and assayed for acid-precipitable radioactivity.

Table 2. Effect of acetyl kidamycin on the processing of poly(A)-containing RNA from nuclei to cytoplasm

Acetyl kidamycin (μg/ml)	Nuclear poly(A)-containing RNA (cpm)	Cytoplasmic poly(A)-containing RNA (cpm)	% Processing of poly(A)-containing RNA (cpm)
0	3471.1	291.6	8.4
1	3471.6	274.3	7.9
10	3471.6	277.7	8.0

HeLa cells (1.0×10^7) were incubated with actinomycin D (0.04 μg/ml) for 30 minutes, then RNA was labeled with [5-^{3}H]uridine. After 10 minutes, the labeling was stopped by addition of 5 μg/ml of actinomycin D, and the culture was divided to 4 portions. One portion was used immediately for extraction of nuclear RNA. To two portions, acetyl kidamycin was added at the indicated concentrations. These two and the final portion were incubated for an additional hour, and RNA was extracted from each cytoplasmic fraction. Isolation and purification of poly(A)-containing RNA were carried out by the same methods as in Table 1.

$$\%\ \text{processing of poly(A)-containing RNA} = \frac{\text{cts/min of cytoplasmic RNA}}{\text{cts/min of nuclear RNA}} \times 100$$

was less than that of 18S in both control and drug-treated cells.

The appearance of radioactive cytoplasmic mRNA is completely inhibited by a low level of acetyl kidamycin, at a concentration of 0.1 μg/ml as shown in Fig. 3. In whole cells, over 90% of HnRNA is destroyed within the nucleus, although a recent evidence[14] suggests that a portion of the RNA adjacent to the poly(A)-sequence at the 3'-end is conserved and passes into the cytoplasm as mRNA.

The effect of acetyl kidamycin on mRNA synthesis and processing was further confirmed by using poly(U)-Sepharose. Acetyl kidamycin strongly inhibits the synthesis of poly(A)-containing RNA in nuclei (Table 1), while processing in the cytoplasm is unaffected even at a concentration of 10 μg/ml. (Table 2)

Discussion

Many antibiotics, which inhibit the synthesis of RNA in mammalian cells, have been found. Among these antibiotics, actinomycins, anthracyclines and chromomycin-like antibiotics bind strongly to DNA,[15] consequently inhibit DNA-dependent RNA synthesis.[16,17,18,19,20] As previously reported,[2,21] acetyl kidamycin binds to DNA and inhibits the incorporation of uridine and thymidine into RNA and DNA, respectively, in HeLa cells. In the present investigation, it was shown that

acetyl kidamycin inhibits the biosynthesis of pre-rRNA, HnRNA and small molecular weight RNA. However, the processing of 45S pre-rRNA into 18S and 28S rRNA and that of HnRNA into mRNA are not affected. It was also observed that the synthesis of HnRNA or mRNA is more sensitive to acetyl kidamycin than that of pre-rRNA.

Several distinct RNA polymerase activities have been identified associated with mammalian cell nuclei. These have been designated as polymerase I, responsible for the transcription of ribosomal RNA,[22,23] and polymerase II, the major nucleoplasmic polymerase responsible for transcribing most of the HnRNA.[21,23] In addition, small molecular weight RNAs are transcribed by a polymerase activity which is distinct from polymerase II.[25,26,27] Polymerase I activity is insensitive to α-amanitin, while polymerase II activity is very sensitive. On the other hand, the synthesis of nucleolar RNA (polymerase I) is more sensitive to actinomycin D than that of HnRNA (polymerase II).

In this respect, our results show that acetyl kidamycin may have a similar action to α-amanitin on RNA synthesis in HeLa cells, though the results are not conclusive.

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