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Inhibition of ribosomal RNA maturation in Friend erythroleukemia cells by 5-fluorouridine and toyocamycin

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Several analogues of purine and pyrimidine bases and nucleosides are rapidly converted *in vivo* to the respective nucleoside-5′-triphosphates and incorporated into RNA chains [1, 2]. Experiments with 5-fluoropyrimidines revealed that with a variety of normal and tumor cells, incorporation of 5-fluorouridine into RNA does not alter appreciably transcription, processing and nucleo-cytoplasmic transfer of mRNA and tRNA, while the formation of mature ribosomes is blocked [3–8]. The preferential block in ribosome formation by 5-fluoropyrimidines could be due to altered conversion of uridine into pseudouridine, an early and essential step in pre-rRNA maturation [9, 10]. On the other hand, a preferential block in ribosome formation was observed also with some other pyrimidine or purine analogues (see [9]).

In the present work we analyzed comparatively the effect of a pyrimidine (5-fluorouridine) and a purine (toyocamycin) analogue on the synthesis and maturation of pre-rRNA in order to clarify whether the block in ribosome formation may be related to a specific base substitution in pre-rRNA chains.

Materials and Methods

Cell cultures, labelling and treatment with analogues. Friend erythroleukemia cells, clone 745, were grown in suspension cultures at 37°C in a minimal essential medium (Gibco), supplemented with 10% fetal calf serum, to a density of 1 to 2 × 10^6 cells/ml. The cells were harvested by centrifugation, resuspended in the same medium at about 10-fold higher concentration and labelled with 1 or 10 μCi/ml of [^{3}H]adenosine or [^{3}H]uridine, or with 2.3 μCi/ml of [^{14}C]uridine. Toyocamycine or 5-fluorouridine at concentrations of 0.5 to 10 μg/ml were added to the same suspension of cells.

Isolation and analysis of RNA. Cytoplasmic and nucleolar RNA were obtained by subsequent extraction with phenol, saturated with 0.14 M NaCl (pH 6.0), at 4° and 50° [11]. The RNA in the water phase was deproteinized two-fold with phenol–0.14 M NaCl and once with phenol:chloroform (1:1, v/v) and precipitated with 2.5 vol. of 96% ethanol, containing 0.14 M sodium acetate, at −20° overnight. The RNA samples were fractionated by agar–urea gel electrophoresis according to Dudov *et al.* [12]. The dried gels were scanned at 260 nm to locate the RNA components. The radioactivity was determined by scanning of the radioautograms at 550 nm or by slicing the dried gels, treatment with 3% NH_4OH overnight, and counting in a Packard, Model 3320, liquid scintillation spectrometer, with a mixture containing 2 vol. of PPO-POPOP-toulene phosphor and 1 vol. of Triton-X 100 [11, 12].

Materials. Analytical grade reagents were used throughout. Toyocamycin was a generous gift by Dr. H. B. Wood, Drug Development Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md., U.S.A., while 5-fluorouridine was obtained from Calbiochem. Lucern, Switzerland. The labelled compounds were a product of the Radiochemical Centre, Amersham, U.K.

Results and Discussion

Addition of 5-fluorouridine or toyocamycin results in a rapid inhibition of the labelling of total cell RNA. The established dose-dependence (Fig. 1)

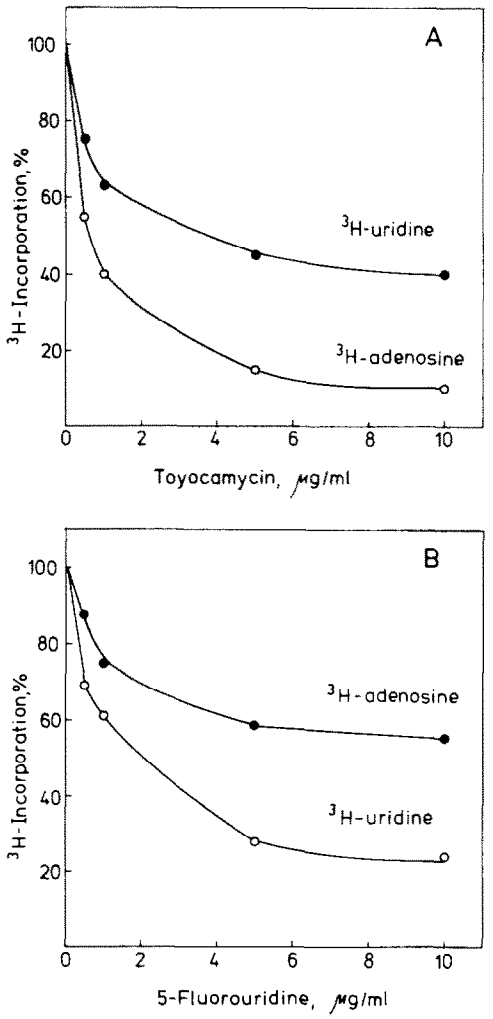

Fig. 1. Dose-dependence of the effect of (A) toyocamycin and (B) 5-fluorouridine on the incorporation of [^{3}H]uridine or [^{3}H]adenosine into total cellular RNA. The analogues are added to a suspension of erythroleukemia cells and after 15 min the cells are labelled for 60 min at 37° with 1 μCi/ml of [^{3}H]uridine or [^{3}H]adenosine.

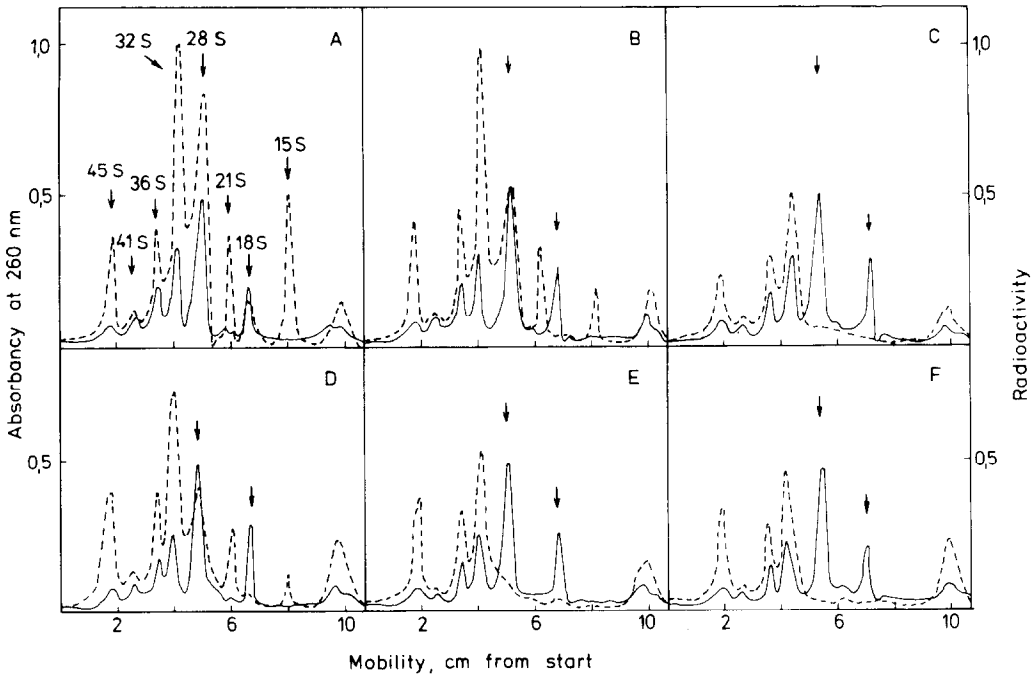

Fig. 2. Agar–urea gel electrophoresis of nucleolar RNA from control cells (A) and cells treated with 0.3 (B) and 3.0 (C) μg/ml of 5-fluorouridine or with 0.3 (D), 3.0 (E) and 5.0 (F) μg/ml of toyocamycin. The labelling is with 2.3 μCi/ml of [^{14}C]uridine for 120 min. The analogues are added 15 min before the radioactive precursor.——, Absorbancy at 260 nm; – – –, Radioactivity recorded from the autoradiogram at 550 nm.

reveals that the inhibitory action is fully displayed at concentrations of 5 μg/ml (corresponding to about 20 μM) of either 5-fluorouridine or toyacamycin. Plateau levels are attained at 40 to 60 per cent inhibition suggesting a differential sensitivity for the different cellular RNA species. The higher inhibition of [^{3}H]adenosine incorporation by toyocamycin and of [^{3}H]uridine by 5-fluorouridine reflects most likely the dilution of the labelled precursor nucleoside-5′-triphosphates by the respective analogue-containing nucleotide [8].

Analysis of labelling of nuclear and cytoplasmic RNA species revealed in agreement with previous studies (see ref. 9), that the observed inhibition of total RNA labelling reflects mainly the inhibition of rRNA labelling, while that of hnRNA, mRNA and tRNA is only sightly affected (data not shown).

In order to elucidate the site of action of toyocamycin and 5-fluorouridine on rRNA synthesis and processing in Friend erythroleukemia cells, comparative studies with the two analogues were carried out. The results from the analysis of nucleolar pre-rRNA and rRNA upon inhibition with either 5-fluorouridine or toyocamycin are presented in Fig. 2. As can be seen, the label in control cells is confined to well delimited 45 S, 41 S, 36 S, 32 S and 21 S pre-rRNA as well as to 28 S and 18 S rRNA. The simultaneous existence of labelled 36 S, 32 S and 21 S pre-rRNA shows that processing of pre-rRNA in Friend erythroleukemia cells proceeds along alternative pathways as found earlier with mouse and rat liver [13, 14], mouse L cells [15] and human lymphocytes [16]. Analysis of the inhibitory action of 5-fluorouridine and toyocamycin reveals that the two analogues have a similar effect on the synthesis and processing of pre-rRNA. This effect may be summarized as follows: (a) The labelling of 45 S pre-rRNA is not appreciably affected, thus showing that analogue incorporation into pre-rRNA chains does not alter the transcription of rRNA genes; (b) the labelling of nucleolar 28 S and 18 S rRNA is practically abolished indicating the occurence of profound alterations in pre-rRNA processing; (c) there is no accumulation of pre-rRNA under blocked formation of mature rRNA, an observation indicating most likely enhanced intranuclear degradation of structurally altered pre-rRNA and preribosomes; (d) the labelling of 36 S pre-rRNA appears to be less affected than that of 32 S pre-rRNA, suggesting a channeling of pre-rRNA processing along alternative routes [8]; (e) a highly labelled 15 S RNA fraction in control cells disappears in parallel with the block of 28 S and 18 S rRNA labelling.

The above results clearly show that incorporation of either 5-fluorouridine or toyocamycin into the polyribonucleotide chain of 45 S pre-rRNA has an identical effect on its processing. The early processing steps are still possible, while the last steps leading to the formation of mature small and large ribosomal subparticles are impossible. This effect may be correlated with a relative accumulation of 36 S pre-rRNA indicating the prevalence of alternative routes of 45 S pre-rRNA processing [8, 14]. It is also noteworthy that a similar early shift of 45 S pre-rRNA processing was observed upon cycloheximide inhibition of protein synthesis [17]. Therefore, the

observed block in ribosome formation is likely to reflect subtle alterations in the structure of preribosomes [18] making impossible the action of processing enzymes, rather than being the consequence of a specific base substitution in the pre-rRNA chain.

Summary. The effect of a pyrimidine (5-fluorouridine) and a purine (toyocamycin) analogue on the synthesis and maturation of precursors to rRNA is studied. Both 5-fluorouridine and toyocamycin do not alter appreciably the synthesis and processing of mRNA and tRNA. The transcription of rRNA genes is also unaltered at concentrations below 3 μg/ml. The processing of 45 S pre-rRNA is still possible, with the prevalence of alternative routes leading to the formation of 36 S pre-rRNA. However, the formation of mature nucleolar 28 S and 18 S rRNA and the appearance of new ribosomes in the cytoplasm is blocked. The similarity in the site of action of these two different analogues suggests that the block in ribosome formation is due to alterations in the conformation of preribosomes making impossible the last steps of their processing.

Institute of Molecular Biology
Bulgarian Academy of Sciences
1113 *Sofia, Bulgaria*

KRASSIMIRA V. HADJIOLOVA
ZLATINA G. NAYDENOVA
ASEN A. HADJIOLOV

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Active efflux common to vincristine and daunorubicin in vincristine-resistant P388 leukemia

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To date, cross-resistance between DNA intercalaters and vinca alkaloids has been observed with a variety of experimental tumor lines [1–7]. With regard to biochemical mechanism of resistance and cross-resistance in adriamycin(ADR)-resistant P388 leukemia, we proposed an increased active efflux of not only anthracyclines [8] but actinomycin-D (ACT) and vinca alkaloids [9]. Danø [10] and Skovsgaard [11] also reported the similar observation with daunorubicin-resistant Ehrlich carcinoma cells.

Since P388/ADR cells possess an efflux system common to DNA intercalaters and vinca alkaloids, it is most likely that vincristine (VCR)-resistant cells are also able to exclude anthracyclines as well as VCR using the similar transport system. On this subject, Skovsgaard [12] already reported that VCR-resistant Ehrlich carcinoma cells possess an energy-dependent drug extrusion common to VCR and daunorubin (DAU). Here we present evidence that P388/VCR cells are also endowed with enhanced capacity for outward transport of those different classes of drug.

Materials and methods. A vincristine-resistant P388 subline (P388/VCR) was established by in vivo procedure, as reported previously [13]. In brief, the resistant subline cells were selected by daily treatment (day 1–9) with 0.25 mg/kg of VCR only on the first transplant generation. *In vitro* sensitivity was determined by the primary suspension culture technique, which was described in the previous paper [8].

For the measurement of drug uptake, cells were harvested 7 days after transplantation of 10^6 cells of each leukemia line into CD2F1 mice, and washed 2 to 3 times and then suspended in Hanks' balanced salt solution (HBSS). Final cell density was adjusted to be 10^6 cells/ml.