INHIBITION OF CHICKEN MYELOBLASTOSIS RNA POLYMERASE II ACTIVITY IN VITRO BY *N*-TRIFLUOROACETYLADRIAMYCIN-14-VALERATE

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1. Introduction

The anthracycline antibiotic adriamycin is a new, yet widely used, antineoplastic drug. It has proved effective in the treatment of various solid tumors as well as acute leukemias [1]. The cytotoxic effect of adriamycin has been correlated with its potent inhibition of cellular DNA and RNA synthesis, presumably by the mechanism of intercalation of the drug molecule into the double helix of DNA and hence interference with the DNA template activities [2–5]. We have found that adriamycin could inhibit the activity of DNA-dependent RNA polymerase II of chicken myeloblastosis (leukemic) cells [6]. This enzyme used denatured DNA, instead of native DNA, as a preferred template for in vitro RNA synthesis [7]. Since denatured DNA would provide fewer intercalation sites for the drug, it would appear that intercalating to DNA template might not be the only mechanism by which adriamycin exerts its inhibitory effect on the leukemic RNA synthesis. This hypothesis has subsequently been substantiated by the following observations [6]:

(1) The template activity of single-stranded DNA purified from denatured calf thymus DNA through two consecutive hydroxyapatite column chromatographies, appeared qually as sensitive to adriamycin inhibition as denatured DNA;
(2) The single-stranded DNA thus purified bound little, if any, adriamycin, as revealed by sucrose gradient centrifugation studies.

Abbreviations: AD 32, *N*-trifluoroacetyladriamycin-14-valerate; NP40, Nonidet P40

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These findings prompted further pursuit of the biochemical mechanisms by which the anthracycline compounds cause inhibition of RNA synthesis. This report describes the effect of *N*-trifluoroacetyladriamycin-14-valerate (an adriamycin derivative) on leukemic RNA synthesis, suggesting that the inhibition of RNA synthesis by anthracycline compounds may involve a direct interaction of the drug and the enzyme RNA polymerase.

2. Experimental procedures

2.1. *Materials*

Unlabeled nucleoside triphosphates were purchased from P-L Biochemicals. [^{3}H]UTP was obtained from New England Nuclear. *Escherichia coli* RNA polymerase (fraction IV) was purchased from Grand Island Biological Co. Calf thymus DNA was from Sigma Chemical Co. Denatured calf thymus DNA was prepared by heating the native DNA at 100°C for 6 min and quick-cooling in an acetone–dry ice bath. Adriamycin (doxorubicin hydrochloride) was manufactured by Farmitalia SPA (Italy) and supplied by Adria Lab. AD 32 was a gift from Dr M. Israel of the Sidney Farber Cancer Institute, Boston MA, who first prepared this compound in late 1973. Large quantities of AD 32 were generously provided by Drs A. Vigevani and F. Arcamone, Farmitalia, Milan. Nonidet P40 was obtained from Shell Chemical Co.

2.2. *Preparation of chicken myeloblastosis RNA polymerase II*

Chicken myeloblastosis (leukemic) cells were prepared and isolated from peripheral blood as in

[8] and frozen at −70°C before grinding. The frozen cells were ground with dry ice using a pre-chilled mortar and pestle and stored at −70°C until use. DNA-dependent RNA polymerase II was isolated from the nuclei of the leukemic cells and purified through DEAE–Sephadex column chromatography and 10–40% glycerol gradient centrifugation as in [7]. The purified enzyme contains no nuclease activity and under standard conditions, the enzyme activity remains linear for ≥60 min [7]. RNA polymerase IIa, which represents the major species of RNA polymerase II in chicken myeloblastosis cells [7], was used here.

2.3. *Assay for RNA polymerase activity*

The reaction mixture (0.1 ml) for the assay of chicken myeloblastosis RNA polymerase II contained 50 mM (pH 7.9) Tris–HCl, 2 μg pyruvate kinase, 4 mM phosphoenol pyruvate, 1 mM $MnCl_2$, 10 mM KCl, 1 mM dithiothreitol, 0.2 mM each of ATP, GTP, and CTP, 0.04 mM UTP and [^{3}H]UTP (1000 cpm/pmol), 10 μg denatured calf thymus DNA, and 2 μg RNA polymerase. The reaction mixture for *E. coli* RNA polymerase was similar to that above except that it contained *E. coli* RNA polymerase, 4 mM $MgCl_2$, 5 mM dithiothreitol, and native calf thymus DNA (10 μg). After incubation for 45 min at 37°C, the reactions were stopped by adding 0.1 ml cold 0.1 M sodium pyrophosphate (adjusted to pH 7.0) containing RNA, 2 mg/ml, bovine serum albumin, 2 mg/ml, 5 mM UTP, and 30% trichloroacetic acid, 0.5 ml. Acid-precipitable radioactivity was collected on Whatman GF/C filters and washed >10 times with 5% trichloroacetic acid. Filters were then dried and counted in a scintillation counter.

2.4. *Cosedimentation of DNA and the anthracyclines through glycerol gradient*

Denatured calf thymus DNA (1 mg/ml) was incubated overnight with adriamycin (0.3 mg/ml) or AD 32 (0.3 mg/ml) in 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM EDTA and 3% NP40. After incubation, 0.25 ml of the mixture was layered onto a 5–20% (v/v) glycerol gradient in 50 mM potassium phosphate buffer (pH 8.0), 1 mM EDTA and 2.5% NP40. The gradients were centrifuged at 49 000 rev./min for 6 h at 4°C in an SW50.1 rotor. The anthracycline alone (without DNA) was processed like the samples containing the drug and DNA and centrifuged in a parallel gradient. After centrifugation, fractions were collected from the bottom of the gradient and diluted with 0.4 ml water. Each fraction was assayed for its anthracycline and DNA content. The anthracycline was determined by A_{480} and the DNA by the diphenylamine method [9].

2.5. *Fluorescence studies*

The effects of DNA on the fluorescent emission spectra of the anthracyclines were studied as follows: adriamycin (40 μg/ml) or AD 32 (40 μg/ml) was incubated overnight with or without denatured calf thymus DNA in 50 mM potassium phosphate buffer (pH 8.0) containing 1 mM EDTA and 2.5% NP40. The fluorescent emission spectrum of the mixture was then determined with a Perkin Elmer MPF-44A fluorescence spectrophotometer at an excitation wavelength of 467 nm.

3. Results and discussion

The structural difference between adriamycin and its derivative, AD 32, is shown in fig.1. With the trifluoroacetyl group replacing the free amino group of the glucosamine of adriamycin, AD 32 was expected not to bind to DNA, since a free glycosidic amino group is needed for DNA intercalation to occur [10,11]. Despite the general consideration deduced from structure–activity studies [12] that the antitumor activity of the anthracycline compounds parallels their abilities to bind to DNA, AD 32 in experimental animal tumor systems and tissue culture studies has significantly greater antitumor activity than adriamycin [11,13]. Initial clinical studies on AD 32 administered to patients with metastatic solid tumors have also been reported with encouraging results [14]. However, the mechanism of its cytotoxic action is not yet known. To determine the effect of AD 32 on in vitro RNA synthesis, AD 32, being insoluble in aqueous solutions, was initially solubilized in NP40. The solvents (Tween 80, ethanol or dimethyl sulfoxide) used in [13,15] for cell-culture studies on AD 32 could not be adopted in our system because we found that they were either detrimental to the enzyme RNA polymerase or produced inconsistent results. NP40, up to final conc. 10%, was found not to affect the enzyme activities (not shown). The data in fig.2 show that AD 32 indeed inhibited the transcriptive activity of leukemic RNA polymerase II and the inhibition was dose-responsive. The relatively high

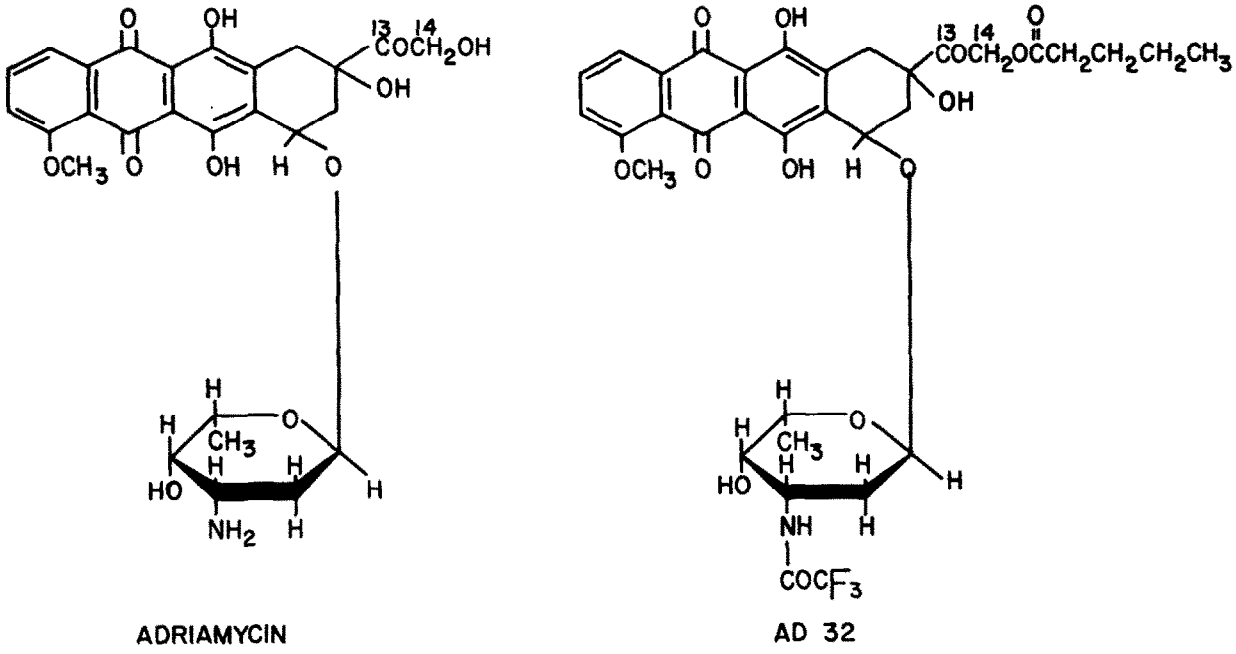
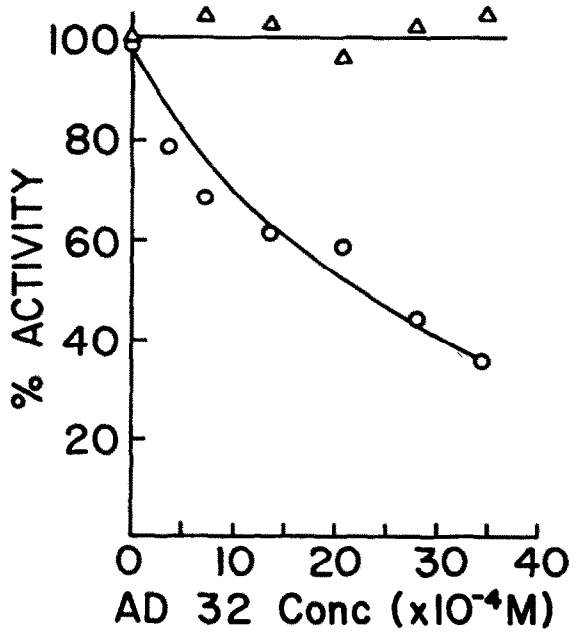


Fig.1. Structural relationship of adriamycin and AD 32.

concentration of AD 32 needed to produce 50% inhibition of RNA polymerase activity in comparison to adriamycin inhibition [6] may be explained by the effect of the solvent, since we found that the inhibitory activity of adriamycin itself on RNA synthesis was considerably (≥5-fold) reduced if the drug was dissolved in NP40. Interestingly, *E. coli* RNA polymerase, assayed under the same conditions, appeared insensitive to AD 32 inhibition (fig.2). The results indicate selectivity of AD 32 inhibition of RNA transcription in eukaryotic versus prokaryotic cells.



The data in fig.3 show that to manifest the inhibition, AD 32 must be preincubated with the enzyme, leukemic RNA polymerase II, for a certain period of time before reaction was initiated. The longer the time AD 32 and the enzyme were preincubated, the greater the inhibition of RNA synthesis was observed. The higher inhibitory activity observed for

Fig.2. Effect of AD 32 on DNA-dependent RNA polymerase activities: Chicken myeloblastosis RNA polymerase II (○) or *E. coli* RNA polymerase (△) was assayed for activity in the presence of various concentrations of AD 32 as indicated. AD 32 was dissolved in NP40 and the final concentration of NP40 in each reaction mixture was adjusted to be 12.5%. RNA polymerase was preincubated with various concentrations of AD 32 or NP40 (for control) at 0°C for 24 h and RNA synthesis was initiated by adding the rest of the reaction mixture. The other assay conditions were as in section 2. Control (100%) activities were 8.5 pmol and 7.5 pmol, respectively, for leukemic and *E. coli* RNA polymerase.

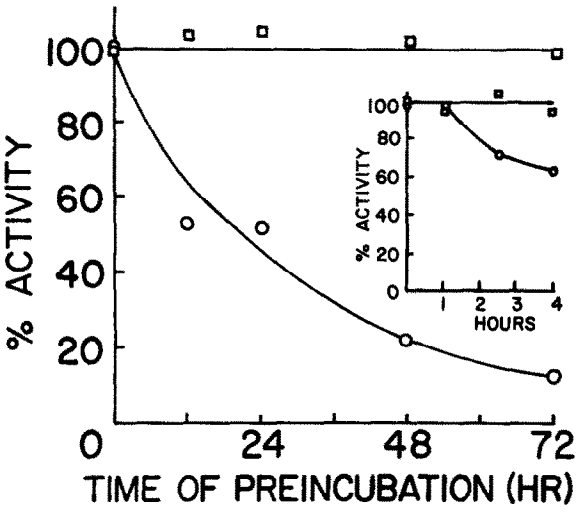


Fig.3. Preincubation studies: Chicken leukemic RNA polymerase II was assayed in the presence of 6.9×10^{-4} M AD 32. The reaction mixture and assay conditions were similar to those in fig.2, except that the final concentration of NP40 in each reaction mixture was 2.5%, and that RNA polymerase (○) or template DNA (□) was preincubated with AD 32 for the time indicated on the abscissa. The control activities for each point on the graph were values obtained when RNA polymerase or template DNA was preincubated with NP40 for the indicated time.

6.9×10^{-4} M AD 32 after 24 h preincubation with the enzyme as compared to the results of fig.2, was owing to the lower concentration of NP40 present in the assay system. The data in fig.3 also show that preincubation of AD 32 with DNA template caused no inhibition of RNA synthesis. These results were what we would expect if the inhibition of leukemic RNA synthesis by AD 32 was indeed not a consequence of DNA-binding or the formation of a DNA-binding compound under our assay conditions. The drug–DNA binding was therefore studied by cosedimentation of the drug and DNA through glycerol gradient by a method similar to that in [6], except that glycerol was used instead of sucrose in the gradient due to the presence of NP40 which caused considerable precipitation in the solution containing sucrose. The results in fig.4 show no indication of binding of AD 32 to the template DNA (denatured calf thymus DNA), while under the same conditions, adriamycin, which was dissolved in NP40, bound to the template in the same manner as it was in an aqueous solution [6]. Furthermore, we found that (fig.5) addition of denatured calf thymus DNA to adriamycin–NP40 solutions caused significant fluorescence quenching, whereas the same DNA had no effect on the fluorescence spectra of AD 32. These observations strongly exclude the consideration DNA-binding as a mechanism for AD 32 inhibition of RNA synthesis. On the other hand, these results clearly suggest that the inhibition of in vitro RNA synthesis by RNA polymerase II involves a drug–

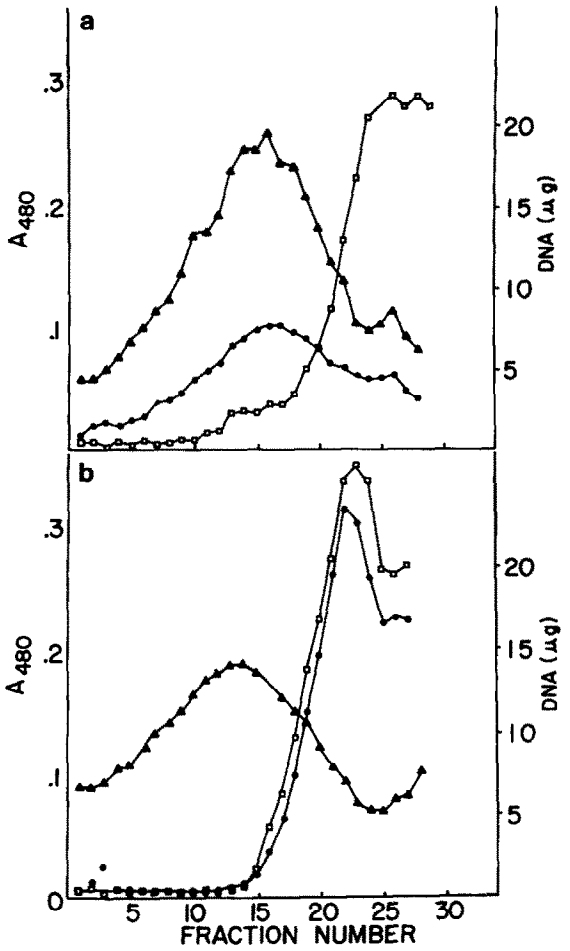


Fig.4. Binding of the anthracyclines to DNA: Denatured calf thymus DNA and the anthracycline compound adriamycin or AD 32 were layered onto a 5–20% glycerol gradient and centrifuged at 49 000 rev./min for 6 h as in section 2. (a) Adriamycin (●) and DNA (▲): the cosedimentation of adriamycin and DNA in the same gradient indicates binding of the drug to DNA. (b) AD 32 (●) and DNA (▲): the non-association of AD 32 and DNA in the same gradient indicates that binding does not occur between AD 32 and DNA. The curve shown (□) indicates A_{480} of adriamycin (a) or AD 32 (b) in the gradient with no DNA added.

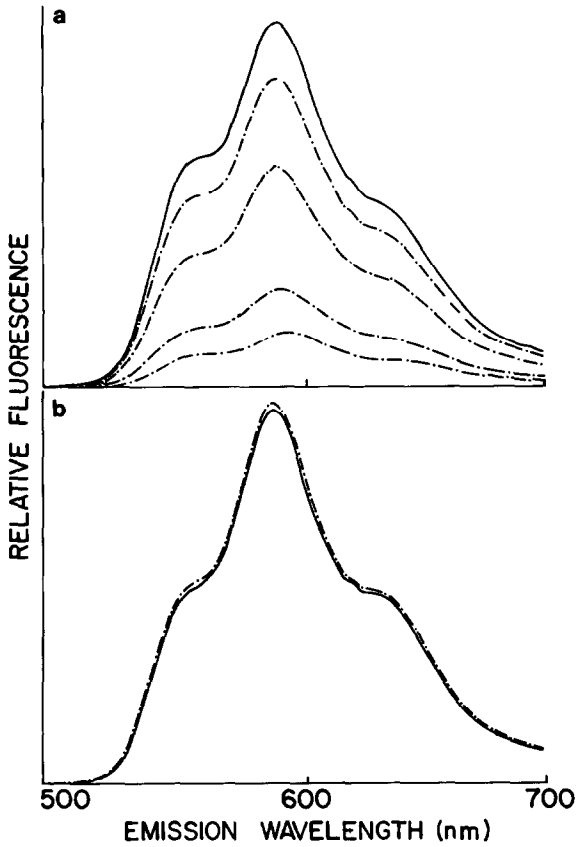


Fig.5. Effect of DNA on the fluorescent emission spectra of the anthracyclines. (a) Emission spectra of adriamycin (——) and adriamycin after mixing with DNA (–·–): the DNA contents were (in the descending order of the graph) 40, 80, 160 and 320 μg/ml. (b) Emission spectra of AD 32 (——) and AD 32 after mixing with DNA, 320 μg/ml (–·–).

enzyme interaction (fig.3). The inhibition at the level of the polymerizing enzyme rather than DNA template, together with our observations that AD 32 does not inhibit in vitro DNA-dependent DNA polymerase activity (unpublished), would probably explain why there is a differential effect of anthracyclines on RNA versus DNA synthesis [16]. Further studies on the binding of AD 32 to RNA polymerase and the effects of other adriamycin derivatives on RNA polymerase activity are in progress.

The above studies provided new insight into the mechanism of the drug action of anthracycline antibiotics. The template activity of the single-stranded calf thymus DNA, which did not bind the drug, was noted to be sensitive still to adriamycin inhibition [6]. These studies reveal that a derivative of adriamycin which was deficient in DNA binding, could also inhibit the in vitro RNA synthesis, and the inhibitory activity depended on the degree of drug–enzyme interactions. They suggest that the tetracycline ring structure of the anthracycline compounds may also contribute to the cytotoxic activities of the drugs. A new biochemical mechanism for the antitumor activity of the anthracycline antibiotics may be proposed: these compounds may alter or bind to the nucleic acid polymerizing enzymes and subsequently reduce the cellular nucleic acid biosynthesis.

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