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INTERACTION OF *N*-TRIFLUOROACETYLADRIAMYCIN-14-*O*-HEMIADIPATE WITH CHICKEN LEUKEMIA RNA POLYMERASE

FORMATION OF DRUG–ENZYME COMPLEX*

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Abstract—The biochemical mechanism of the *N*-trifluoroacetyladriamycin-14-*O*-hemiadipate-induced inhibition of RNA synthesis *in vitro* by chicken (myeloblastosis) leukemia RNA polymerase II was studied. The inhibition was found to be dependent upon preincubation of the drug with the enzyme prior to enzyme assays, suggesting that drug–enzyme interactions occur. A drug–enzyme association complex was subsequently isolated through glycerol gradient sedimentation and further characterized by fluorescent microscopic studies. The drug was dissociated from the complex upon sodium dodecyl sulfate (SDS)-gel electrophoresis, revealing the non-covalent nature of the binding between the drug and the RNA polymerase.

N - Trifluoroacetyladriamycin - 14 - *O* - hemiadipate (AD 143‖), a newly synthesized adriamycin derivative (Fig. 1), has been demonstrated to be therapeutically superior to adriamycin in terms of its greater antitumor activity and lower cardiocytotoxicity [1]. Because of its high water solubility, AD 143 is preferred to its lipophilic and water-insoluble analog AD 32 as a candidate for clinical applications. Since fluorescence spectroscopic studies show that there is no interaction between AD 143 and DNA [2], the biochemical mechanism of the antitumor activity of AD 143 may be different from that generally considered for adriamycin and other anthracycline compounds, namely inhibition of both DNA and RNA syntheses primarily through DNA-binding [3–5]. We found previously that AD 143 has differential effects towards both RNA versus DNA synthesis and eukaryotic versus prokaryotic RNA synthesis [2]; AD 143 inhibited the activity of eukaryotic RNA polymerases and had little effect on the activity of DNA polymerases or prokaryotic RNA polymerases. The inhibition of eukaryotic RNA synthesis by AD 143 was found to be related to its ability to prevent the formation of a stable RNA polymerase–DNA complex for RNA chain initiation [6]. The present studies describe our experimental observations, which suggest that AD 143 may form a drug–enzyme complex with RNA polymerase to cause the observed inhibition of RNA chain initiation.

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‖ Abbreviations: AD 143, *N*-trifluoroacetyladriamycin-14-*O*-hemiadipate; AD 32, *N*-trifluoroacetyladriamycin-14-valerate and IC_{50} value, concentration producing 50% inhibition.

MATERIALS AND METHODS

Materials. Unlabeled ribo- and deoxyribonucleoside triphosphates were purchased from P-L Biochemicals (Milwaukee, WI). [^{3}H]UTP was obtained from New England Nuclear (Boston, MA). AD 143 was synthesized in the laboratory of one of the authors' (M.I.) according to procedures described elsewhere [1]. Solutions of the drug were freshly prepared immediately before use. Calf thymus DNA was obtained from the Sigma Chemical Co. (St. Louis, MO). Denatured calf thymus DNA was prepared by heating the native DNA at 100° for 10 min followed by quick cooling.

Preparation and assay of chicken myeloblastosis RNA polymerase II. RNA polymerase II was isolated from the nuclei of chicken leukemia (myeloblastosis) cells and purified as described [7]. The DEAE-Sephadex column used in the purification procedure resolves two subspecies of the enzyme (IIa and IIb). Enzyme IIa, which represents the major subspecies [8], was used for the present studies. The purified RNA polymerase IIa showed a single polypeptide band when subjected to polyacrylamide gel electrophoresis under non-denaturing conditions, indicating the homogeneity of the enzyme preparations. The enzyme (2 μg/ml) was assayed in a 0.1-ml reaction mixture containing 50 mM Tris–HCl (pH 7.9), 2 μg pyruvate kinase, 4 mM phosphoenolpyruvate, 1 mM $MnCl_2$, 10 mM KCl, 1 mM dithiothreitol (DTT), 0.2 mM each of ATP, GTP, and CTP, 0.04 mM UTP and [^{3}H]UTP (600–700 cpm/pmole) and 10 μg of denatured calf thymus DNA. One unit of activity is defined as 1 nmole of [^{3}H]UMP incorporated in 30 min at 37°, and the specific activity of the newly purified RNA polymerase was 303 units/mg. To determine the effect of AD 143 on RNA polymerase II activity, RNA polymerase II was pre-

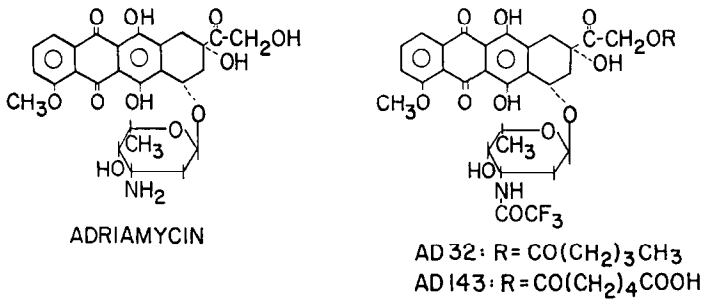

Fig. 1. Structural relationship between adriamycin and its derivatives, AD 143 and AD 32.

incubated with buffer (10 mM Tris–HCl, pH 7.9; as controls) or AD 143 (dissolved in buffer) at 0° for the time indicated in each experiment prior to the addition of other reactants. After incubation for 45 min at 37°, the reactions were stopped by adding 0.1 ml of cold 0.1 M sodium pyrophosphate (pH adjusted to 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 more than ten times with 5% trichlororacetic acid. Filters were then dried and counted in a scintillation counter.

Glycerol gradient sedimentation of AD 143–RNA polymerase II complex. Purified RNA polymerase II (0.1 to 0.3 mg) was concentrated by adding solid ammonium sulfate to 65% saturation followed by centrifugation at 114,000 *g* for 45 min in an SW 50.1 rotor. The precipitate was resuspended in 0.2 ml of 10 mM Tris, pH 7.9, containing 2 mM dithiothreitol. RNA polymerase II suspension (0.1 ml) was mixed with 0.1 ml of buffer (10 mM Tris, pH 7.9) or buffer containing AD 143 (0.2 mg), and incubated for 3 hr at 0°. The incubation mixture was then sedimented in 5 ml of 0.2 M phosphate buffer, pH 8.0, containing 2 mM DTT, 1 mM EDTA, and a linear 10–40% glycerol gradient by centrifugation at 234,000 *g* for 15 hr in an SW 50.1 rotor at 4°. Buffer containing AD 143 alone was processed simultaneously and run in a parallel gradient to serve as a drug control. Fractions (0.2 ml) were collected from the bottom of the gradients. A portion (0.01 ml) was withdrawn from each fraction and assayed for RNA polymerase activity. Afterwards, 0.3 ml H_2O was added to each fraction, and AD 143 concentrations were measured spectrophotometrically at 480 nm.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Sodium dodecyl sulfate gel electrophoresis was carried out according to the method of Laemmli [9]. Following electrophoresis the gels were fixed and silver stained according to the procedure of Sammons *et al.* [10].

RESULTS

Inhibition of RNA polymerase activity by AD 143 as a function of the time of enzyme–AD 143 preincubation. Chicken myeloblastosis RNA polymerase II was assayed for activity *in vitro* in the presence of various concentrations of AD 143. It was found that the degree of the inhibition of RNA synthesis by AD 143 was in proportion to the length of time that RNA polymerase and AD 143 were preincubated before the reaction was initiated (Fig. 2); thus, with longer preincubation time a lower AD 143 concentration was needed to cause the inhibition. As shown in Fig. 2a, the IC_{50} value for AD 143 induced inhibition of RNA synthesis was 15 μM for a 5-hr preincubation time and decreased to 4 μM if

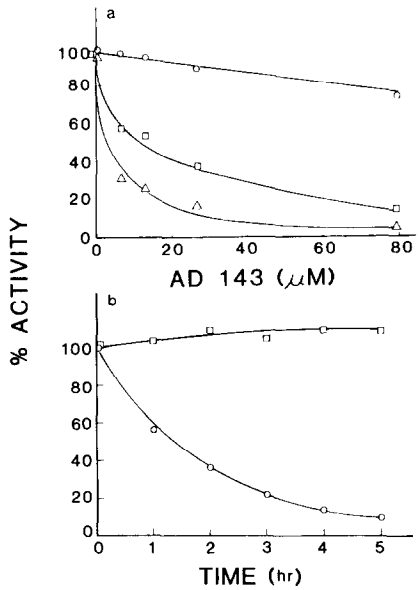

Fig. 2. Effect of AD 143 on chicken leukemia RNA polymerase II activity. (a) Effect of AD 143 concentration on the enzyme activity: chicken myeloblastosis RNA polymerase II was assayed for activity in the presence of various concentrations of AD 143 as indicated. RNA polymerase was preincubated with buffer (10 mM Tris–HCl, pH 7.9, as controls) or buffer containing AD 143 at 0°, and RNA synthesis was initiated by adding the rest of the reaction mixture. The other assay conditions were as described under Materials and Methods. Preincubation time: (○) 0 hr; (□) 5 hr; and (△) 24 hr. Control (100%) activities were 50.1, 19.4 and 14.1 pmoles, respectively, for 0-, 5-, and 24-hr preincubation. (b) Effect of preincubation time on the inhibition: chicken myeloblastosis RNA polymerase II was assayed in the presence of 66 μM AD 143. RNA polymerase (○) or template DNA (□) was preincubated with AD 143 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 buffer for the indicated time.

the duration of the preincubation time was increased to 24 hr. Preincubation of AD 143 with DNA template, on the other hand, caused no effect on the reaction (Fig. 2b). These experiments suggested that the inhibitory effect of AD 143 on RNA synthesis depends upon drug–enzyme interactions.

Formation of AD 143–RNA polymerase binding complex. To determine whether AD 143 and RNA polymerase interactions involve the formation of a drug–enzyme binding complex, RNA polymerase II was preincubated with AD 143 at 0° for 3 hr and centrifuged in a 10–40% glycerol gradient by a procedure described under Materials and Methods (Fig. 3c). RNA polymerase alone (Fig. 3a) and AD 143 alone (Fig. 3b) were processed under the same conditions and centrifuged in parallel gradients. After centrifugation, the enzyme sedimented at fractions 6–10 (Fig. 3a) and the drug at fractions 16–24 with some aggregates sedimenting at a relatively lower position (fractions 8–14) in the gradient (Fig. 3b). When the enzyme and AD 143 were preincubated and centrifuged together in a gradient, we found that the majority of the drug sedimented in the same fractions as the enzyme (Fig. 3c). To argue against the possibility that RNA polymerase and AD 143 were associated nonspecifically in the gradient, AD 143 was preincubated with bovine serum albumin or chicken leukemic DNA polymerase alpha, and the mixture was sedimented in a glycerol gradient under similar conditions. The results showed that AD 143 and bovine serum albumin or DNA polymerase alpha did not co-sediment in the gradient (data not shown), suggesting that the observed association of AD 143 and RNA polymerase may involve the formation of a specific enzyme–drug binding complex.

As an additional control for the experiments shown in Fig. 3, calf thymus DNA was run through the gradients together with AD 143. We have shown previously that addition of DNA to AD 143 solutions has no effect on the fluorescence spectrum of AD 143 [2], and we suggested that there are no interactions between AD 143 and DNA. The results of Fig. 4 (a–c) further demonstrate that AD 143 unlike adriamycin, its parent drug, does not bind to DNA, since adriamycin, if processed in a similar manner, would sediment together with DNA [11]. Therefore, the mechanism of the drug action of AD 143 may involve drug–enzyme, rather than drug–DNA, bindings.

Isolation and fluorescent microscopic studies of AD 143–RNA polymerase binding complex. Fractions 6–10 of gradients a–c and fractions 16–24 of gradient b of Fig. 3 were pooled separately, and tricholoracetic acid was added to each sample to a final concentration of 7%. The mixture was then kept overnight at 4°. Under these conditions, samples containing proteins form precipitates. The solutions were centrifuged and the supernatant fractions were removed. The results showed that only gradients a and c contained precipitates (Fig. 5). The precipitates

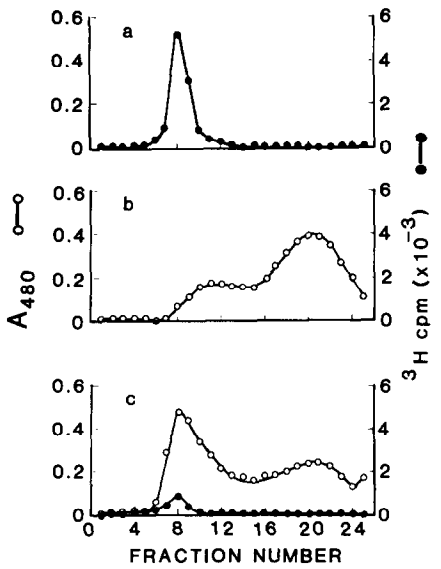

Fig. 3. Binding of AD 143 to RNA polymerase. Chicken myeloblastosis RNA polymerase II and AD 143, after preincubation for 3 hr, were run through a 10–40% glycerol gradient as described under Materials and Methods. (a) RNA polymerase alone; (b) AD 143 alone; (c) RNA polymerase and AD 143: the cosedimentation of AD 143 and RNA polymerase in the same gradient indicates binding of the drug to the enzyme. Key: (○) AD 143 concentration as determined by A_{480} absorbancy (the molar extinction coefficient of AD 143 at 480 nm equals 10,965); and (●) enzyme activity as determined by [^{3}H]UTP incorporation.

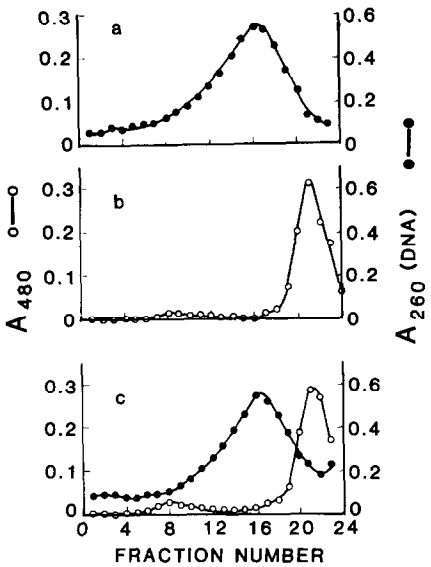

Fig. 4. AD 143–DNA binding studies. Denatured calf thymus DNA (0.2 mg) and AD 143 (0.1 mg) were preincubated at 0° overnight and centrifuged through a 5–20% glycerol gradient in an SW 50.1 rotor at 48,000 rpm for 5 hr by a procedure described previously [11]. After centrifugation, fractions were collected from the bottom of the gradient and diluted with 0.4 ml water. Absorbancies at 480 nm (due to AD 143) and at 260 nm (due to DNA and AD 143) were determined. A_{260} readings shown in the figure were values contributed by DNA only (the A_{260} readings contributed by AD 143 were calculated from the A_{480} readings and have been subtracted). (a) DNA alone; (b) AD 143 alone; (c) DNA and AD 143: the non-association of DNA and AD 143 in the same gradient indicates that binding does not occur between AD 143 and DNA.

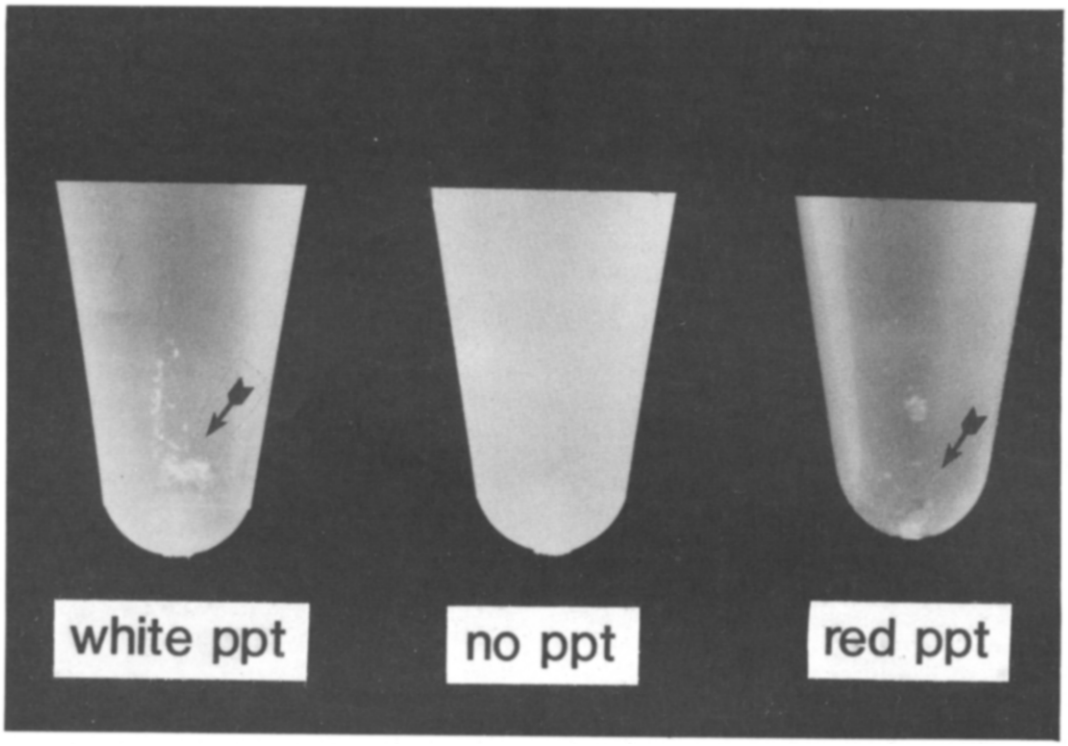

Fig. 5. AD 143–RNA polymerase binding complex. Fractions 6–10 of gradient a–c and fractions 16–24 of gradient b of Fig. 3 were pooled separately and precipitated with 7% trichloroacetic acid as described in the text. Left, RNA polymerase alone (from gradient a) showing white-brownish precipitates; center, AD 143 alone (from either fractions 6–10 or fractions 16–24 of gradient b) showing no precipitates; right, AD 143–RNA polymerase binding complex (from gradient c) showing red-pigmented precipitates. The arrows indicate the precipitates.

of gradient c appeared pigmented, indicating the association of AD 143 (which contains a red-colored aglycone) with RNA polymerase (Fig. 5, right). Furthermore, when the samples were examined under the fluorescence microscope (Zeiss Standard 16FA, Calson Optical Instruments, Inc.), the precipitates of gradient c emitted drug-specific fluorescence (data not shown), a typical characteristic of anthracycline compounds [12].

Electrophoresis of the AD 143–RNA polymerase binding complex. Like the molecular structures of other eukaryotic RNA polymerases that contain multiple subunits [13–15], RNA polymerase II from chicken leukemia cells contained 8 subunits of molecular weights ranging from 24,000 to 220,000 (data not shown). With our initial intent to determine which subunit of the RNA polymerase binds with AD 143, the trichloroacetic acid-precipitated enzyme and enzyme–drug complex isolated from gradients 3a and 3c were subjected to polyacrylamide gel (10% gel) electrophoresis in the presence of sodium dodecyl sulfate [9]. After electrophoresis, the fluorescence of AD 143 was detected and photographed by placing the gel directly on a Foto UV 300 DNA Transilluminator (Fotodyne, Inc.). The subunits of RNA polymerase were located by silver-stain [10], and photographed by placing the gel on a fluorescent light box. The results showed that AD 143 moved faster than the enzyme, and that there was no fluorescence of AD 143 associated with the RNA polymerase subunit region (data not shown). The separation of AD 143 from the polymerase during this procedure suggests that the formation of RNA polymerase–AD 143 complex may involve non-covalent bonds.

DISCUSSION

Adriamycin is an antitumor antibiotic of the anthracycline class that is used widely in the treatment of human neoplastic diseases including acute leukemias and a wide range of solid tumors [16]. Its prolonged use is limited, however, by a dose-limiting cardiotoxicity, which may lead to fatal congestive heart failure [17]. AD 32, a derivative of adriamycin, has been synthesized and reported to be superior to and less toxic than adriamycin [18]. Nevertheless, the use of AD 32 is also limited because of its highly lipophilic nature. AD 143, an analog of AD 32 with improved aqueous solubility, has recently been synthesized and developed in response to the need to overcome clinical formulation and administration difficulties of AD 32 [1]. The water-soluble property of AD 143 allows us to study in depth at the molecular level the biochemical mechanisms of action of this type of adriamycin derivative which has potential clinical value in cancer chemotherapy.

We have reported recently that AD 143 inhibits the activities of RNA polymerase I and II, but not DNA polymerase α, from chicken leukemia cells,

and that AD 143 has no significant effect on either RNA or DNA polymerase of *Escherichia coli* cells [2]. These properties of high selectivity of AD 143 are similar to those we previously reported for AD 32 [11, 19], although the latter compound inhibited RNA synthesis at a relatively high drug concentration. Like AD 32, AD 143 was found to be deficient in DNA-binding (Fig. 4). Therefore, the study of AD 143 may reveal general information regarding the biochemical mechanism of the cytotoxic action of non-DNA-binding derivatives of adriamycin.

AD 143 inhibits leukemia cell RNA synthesis by reducing the frequency of RNA chain initiation; the susceptible step in the initiation process was found to be the formation of stable complexes between RNA polymerase and the DNA template [6]. While AD 143 causes no inhibition of *E. coli* RNA polymerase activity, it was found not to affect the *E. coli* RNA polymerase–template DNA complex formation [6]. In this report we present evidence to show that AD 143 may form a specific binding complex with RNA polymerase. Therefore, it is possible that, as a result of this drug–enzyme binding, RNA polymerase is incapable of forming an initiation complex with DNA, and RNA synthesis is subsequently inhibited. At present, the nature of the binding force of the drug–enzyme complex is not completely clear. The results of electrophoresis of the complex on a SDS-polyacrylamide gel have excluded the possibility of covalent binding. Since inhibition of RNA synthesis by AD 143 cannot be overcome by increasing DNA concentrations of the reaction [6], it appears that AD 143 may bind to RNA polymerase at sites other than the DNA-binding site of the enzyme molecule, and that the drug–enzyme binding may cause an irreversible conformational change of the enzyme molecule and subsequently prevent a normal RNA polymerase–DNA template interaction. While the enzyme–drug interaction may occur readily at high AD 143 concentrations (60 μM), there appears to be a similar conformational change that gradually takes place at lower concentrations of the drug as evidenced by the critical enzyme–drug preincubation time (Fig. 2).

Adriamycin and related anthracyclines have been reported to inhibit nucleic acid synthesis through the formation of an intercalation complex with double-stranded DNA [3–5]. The present studies, which demonstrate that a derivative of adriamycin totally devoid of any form of DNA-binding characteristics inhibits RNA synthesis by inactivating RNA polymerase, suggest a new mechanism for anthracycline anticancer drugs. The fact that AD 143 exhibits inhibition at the level of the polymerase molecule rather than the DNA template may account for the observation that AD 143 appears to have a more selective action toward macromolecule synthesis than its parental drug adriamycin [2]. Consequently, AD 143 may be expected, as has been demonstrated by preclinical studies [1], to cause fewer untoward side effects when used as a cancer chemotherapeutic agent.

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