Dependence of Nucleic Acid Degradation on *in Situ* Free-Radical Production by Adriamycin

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ABSTRACT: Adriamycin (Adr) is one of the most powerful antitumor drugs. Its therapeutic effect may be due to its cyclic reduction–oxidation and, thus, generation of oxygen radicals. Using the spin–trap 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO) and EPR we have demonstrated that in an enzymatic system consisting of NADPH, NADPH-cytochrome P-450 reductase, and $Fe(EDTA)_2$ Adr stimulates formation of •OH radicals in the presence of DNA or RNA with equal efficiency. Incubation of nucleic acids in the Adr-dependent reaction generating •OH radicals resulted in extensive degradation of double- and single-stranded DNA, but did not effect RNA. In contrast, both DNA and RNA were effectively destroyed in a footprinting system, ascorbate–$Fe(EDTA)_2$–H_2O_2, which generates •OH radicals in massive quantities. Fluorescence assays indicated that Adr forms stable complexes with ds- and ss-DNA but reacts only slightly with RNA. We conclude that the formation of Adr–nucleic acid complex is necessary for •OH radical-mediated cleavage of the latter, and thus, Adr may be regarded as a chemical nuclease acting *in situ*.

Adriamycin (Adr) is an antibiotic which is widely used for the therapy of various human cancers (Arcamone, 1981; Young et al., 1981). Two main mechanisms were proposed to explain the biological activity of Adr. The first suggests, based on experimental data, that the drug interacts directly with DNA, leading to the inhibition of both replication and transcription (Pigram et al., 1972; Painter, 1978). Many investigators are now focusing on the thermodynamic and kinetic characteristics of Adr–DNA interactions (Chaires et al., 1982; Eriksson et al., 1988; Chaires, 1990). Recently, a three-dimensional structure of Adr–DNA complex has been obtained (Frederick et al., 1990). The second proposed mechanism suggests that Adr induces an oxygen radical formation which brings about oxidative stress in cells and nucleic acid cleavage.

Indeed, numerous experimental data indicate that the Adr is reduced to a semiquinone radical which is generated in electron-transfer chains and induces the formation of highly reactive hydroxyl radicals (see Powis (1989) and references therein) capable of cleaving both double- and single-stranded DNA molecules (Brawn & Fridovich, 1981; Imlay & Lihn, 1988). Hydroxyl radicals are formed in the Fenton reaction: $H_2O_2 + Fe(II) \rightarrow {}^{\bullet}OH + OH^- + Fe(III)$. The superoxide radical $O_2^{\bullet-}$, which serves as the source of hydrogen peroxide, can be generated in the cell through enzymatic oxidation of xanthine to uric acid (Fridovich, 1970) or during the autooxidation of various reduced NAD(P)H-dependent flavine-containing enzymes. The resistance of some tumor-derived cell lines to Adr appears to be due to enhanced activity of enzymes which catalyze the destruction of hydrogen peroxide and superoxide radicals (Sinha et al., 1987; McGrath et al., 1989; Yin et al., 1989). The *in vitro* enzymatic reduction of Adr by NADPH-cytochrome P450 reductase in the presence of DNA leads to the production of •OH radicals and DNA cleavage (Berlin & Hazeltine 1981; Rumyantseva et al., 1989).

The relationship between these two proposed mechanisms of Adr action, namely, Adr–DNA complex formation and Adr-induced oxygen radical-dependent nucleic acid cleavage, remains unclear. A clarification of this issue is important, not only for understanding the therapeutic and side effects of Adr *per se*, but also for the development of new specific bioreductive drugs (Dikalov et al., 1992).

In this work we examined the ability of Adr to bind to different forms of DNA and RNA molecules and consequently to induce nucleic acid cleavage. The data presented here suggest that complex formation between Adr and nucleic acids precedes and is required for hydroxyl radical-mediated cleavage of the latter.

MATERIALS AND METHODS

Restriction enzymes HindIII, EcoRI, and proteinase K were purchased from Boehringer-Mannheim. Plasmid SP6 DNA, RNasin RNase inhibitor, and DNase I were from Promega. MuLV reverse transcriptase was purchased from BRL. [α-^{32}P]dATP (~3000 Ci/mmol) and [α-^{32}P]UTP (~3000 Ci/mmol) were from Amersham, while RNase A and RNase T1 were from Sigma. Single-stranded DNA of M13mp18 (for fluorescence measurements) was from USB. Purified NADPH-cytochrome P-450 reductase was obtained from Oxford Biomedical Research, Inc. (Michigan). The double-stranded Bluescript plasmid DNA was purchased from Stratagene. Adriamycin hydrochloride, 5,5′-dimethyl-1-pyroline *N*-oxide (DMPO), NADPH, cytochrome c, superoxide dismutase, catalase, and yeast tRNA were obtained from Sigma.

A spin–trap DMPO was purified as described (Buettner & Oberley 1978). The absence of paramagnetic admixtures was determined from the EPR spectrum. The activity of NADPH-cytochrome P-450 reductase was determined by measuring the rate of cytochrome c reduction (Phillips & Langdom 1962).

Plasmids. HindIII-HincII 450 bp BCR-ABL junction containing fragment BCR-ABL cDNA clone K30 (Shtivelman et al., 1985) was subcloned into HindIII-HincII sites of pGEM3 or pGEM4 (Promega).

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Preparation of Radiolabeled (End-Labeled) DNA. Plasmid pGEM3, containing 450 bp of BCR-ABL cDNA, was digested with EcoRI and end-labeled with [α-^{32}P]dATP, using reverse transcriptase. Following phenol/chloroform extraction and ethanol precipitation, DNA was further digested with HindIII restriction endonuclease. To obtain double-stranded end-labeled DNA, the reaction mixture was electrophoresed through 5% PAGE containing 0.1% bis(acrylamide). A 481 bp (31 nucleotides are of pGEM polylinker origin) end-labeled ds-DNA fragment was eluted and used in further experiments. To obtain single-stranded end-labeled DNA, end-labeled DNA was extracted with organic solvents and, following ethanol precipitation, was redissolved in denaturing dye solution (30% DMSO, 1 mM EDTA, 0.05% xylencyanol, 0.05% bromphenol blue) at a concentration of 1 μg of DNA per 40 μL. The mixture was heated at 90 °C for 2 min and electrophoresed through 5% PAGE containing 0.1% bis(acrylamide). A 481 bp end-labeled ss-DNA fragment was eluted.

Preparation of Uniformly-Labeled RNA Substrates. Plasmid pGEM3, containing 450 bp of BCR-ABL cDNA, was transcribed *in vitro* using SP6 RNA polymerase in a reaction volume of 20 μL. The reaction mixture included 4 μL of 5X transcription buffer (Promega), 10 mM DTT, RNasin (1 U/μL), 0.5 mM each of ATP, GTP, and CTP, 12 μM UTP, 50 μCi of [α-^{32}P]UTP, 2 μg of plasmid template DNA, and 10 U of SP6 RNA polymerase and was incubated for 60 min at 37 °C. Following RNA synthesis, DNase was added to a concentration of 1 U/μg of DNA, and incubation was continued for another 15 min. Following extraction with phenol/chloroform, RNA was recovered by ethanol precipitation and redissolved in water. To obtain double-stranded RNA, the RNAs transcribed with SP6 RNA polymerase from pGEM3-BCR-ABL and pGEM4-BCR-ABL were hybridized to each other at 45 °C for 2 h. The hybridization cocktail consisted of 80% formamide, 40 mM PIPES (pH 6.5), 0.4 M NaCl, and 1 mM EDTA. The RNAs were then treated with RNases (2 mg/mL of RNaseA, 0.1 mg/mL of ribonuclease T1 in buffer composed of 10 mM Tris·HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA) for 2 h at 30 °C (until a decrease in incorporated radioactivity was no longer observed). Following proteinase K treatment and extraction with phenol/chloroform, hybrid double-stranded RNA was recovered by ethanol precipitation. To obtain an end-labeled RNA, plasmid DNA was transcribed *in vitro*, as described in protocols of Promega (Riboprobe SP6). Five to ten micrograms of RNA were end-labeled as described (Carter et al., 1990) and used in the adriamycin cleavage reaction, as well as for untreated control.

EPR Study of Radical Formation. •OH radical formation was followed by EPR of the spin adduct, DMPO-OH, formed by the action of an •OH radical. The EPR spectra were measured on a E-12 Varian spectrometer in a flat sealed cell (200 μL volume) at room temperature. The experimental conditions were as follows: sweep width, 100 G, microwave power, 20 mW; modulation amplitude 0.8 G; receiver gain 10^4.

Hydroxyl Radical Cleavage of Nucleic Acids. One μg ($\sim 10^5$ cpm) of end-labeled DNA (ds or ss) or 100 ng ($\sim 10^5$ cpm) of uniformly labeled RNA (ss or ds) were treated with a cocktail containing 100 μM $(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$, 200 μM EDTA, 2 mM ascorbic acid, 0.03% H_2O_2, 50 mM KCl, and 1 mM $CaCl_2$ in 20 mM sodium phosphate buffer (pH 7.4). The reaction proceeded at 37 °C for 30 min in a reaction volume of 20 μL.

NADPH-Dependent Adriamycin Cleavage of Nucleic Acids. One microgram ($\sim 10^5$ cpm) of end-labeled DNA (ss or ds), 100 ng ($\sim 10^5$ cpm) of uniformly labeled RNA (ss or ds), or 2 μg ($\sim 2 \times 10^4$ cpm) of end-labeled RNA was treated with a cocktail containing 10 μM $Fe(Cl)_3$, 20 μM EDTA, 25 mM NaCl, 2 mM NADPH, 0.1 mM adriamycin, and 0.0025 unit of NADPH-cytochrome P-450 reductase in 20 mM sodium phosphate buffer (pH = 7.4) in a reaction volume of 20 μL. When RNA was used, 1 U of RNasin was added to the reaction mixture. Reactions were conducted for 30 min at 37 °C. Following incubation, nucleic acids were extracted with phenol/chloroform, recovered by ethanol precipitation, and analyzed on 7% denaturing urea-PAGE.

Spectral Measurements. Nucleic acid concentrations were determined with a UVIKON 810 spectrophotometer at λ = 260 nm. Adriamycin concentrations were measured spectrophotometrically at λ = 480 nm, ϵ = 11 500 M^{-1} cm^{-1} (Chaires et al., 1982). Fluorescence titration experiments were performed on a Shimadzu RF-540 spectrofluorimeter at ambient temperature. The ratio of the fluorescence intensity of adriamycin in the absence of a nucleic acid (I_0) and in the presence of a nucleic acid (I) was used to calculate the amount of free drug according to the equation (Chaires et al., 1982)

$$C_f = C_T(I/I_0 - P)/(1 - P) \quad (1)$$

where C_T is the known amount of added drug and P is the ratio of the observed quantum yield of fluorescence of the totally bound drug to that of the free drug. A value of P = 0.05 was used (Chaires et al., 1982). The amount of Adr bound to the nucleic acid (C_B) was determined by the difference

$$C_B = C_T - C_f \quad (2)$$

RESULTS

Adriamycin–Nucleic Acid Interaction. Adr exhibits fluorescence at 555 nm (excitation λ = 480 nm) in aqueous solutions. Interaction of Adr with DNA leads to almost complete quenching of the fluorescence (Chaires et al., 1982; Bareello et al., 1988). This enabled quantitative characterization of the drug–nucleic acid interaction. Quenching of Adr fluorescence by single- and double-stranded DNA (M13 and Bluescript, respectively) and by tRNA is shown in Figure 1. The presence of single- or double-stranded DNA is accompanied by quenching of Adr fluorescence to nearly the same extent. In contrast, the presence of tRNA has very little effect. Binding parameters for Adr–DNAs interaction were determined by Scatchard plot analysis (Chaires et al., 1982). A double reciprocal plot allows the calculation of the parameters for the Adr–tRNA interaction (Chien et al., 1977). The binding constant (K) and the number of binding sites (n) of Adr (per mole of nucleotide) for double- and single-stranded DNAs are similar: $K_1 \sim 2 \times 10^6$ M^{-1}, n = 0.2. The analogous numbers for the Adr–tRNA complex were $K_2 \leq 10^3$ M^{-1}, n = 0.06.

Generation of Hydroxyl Radicals. Adriamycin is known to stimulate the formation of hydroxyl radicals during aerobic reduction by NADPH-cytochrome P-450 reductase [in the presence of Fe(III) ions or of Fe(III) complexes in solution (Powis 1989)]. We investigated whether nucleic acids affect the kinetics of Adr-mediated formation of hydroxyl radicals. To define •OH radicals, a DMPO spin–trap was used. As a result of chemical reaction with •OH radical, DMPO forms a spin adduct with a characteristic ESR spectrum, namely a quartet with the component ratio 1:2:2:1 and $a_N = a_H$ = 14.9 G (Buettner & Mason, 1990). The kinetics of spin adduct formation and the magnitude of the maximum amplitude of the ESR signals characterize the rate of redox processes in the system (Rumyantseva et al., 1989; Dikalov, 1992).

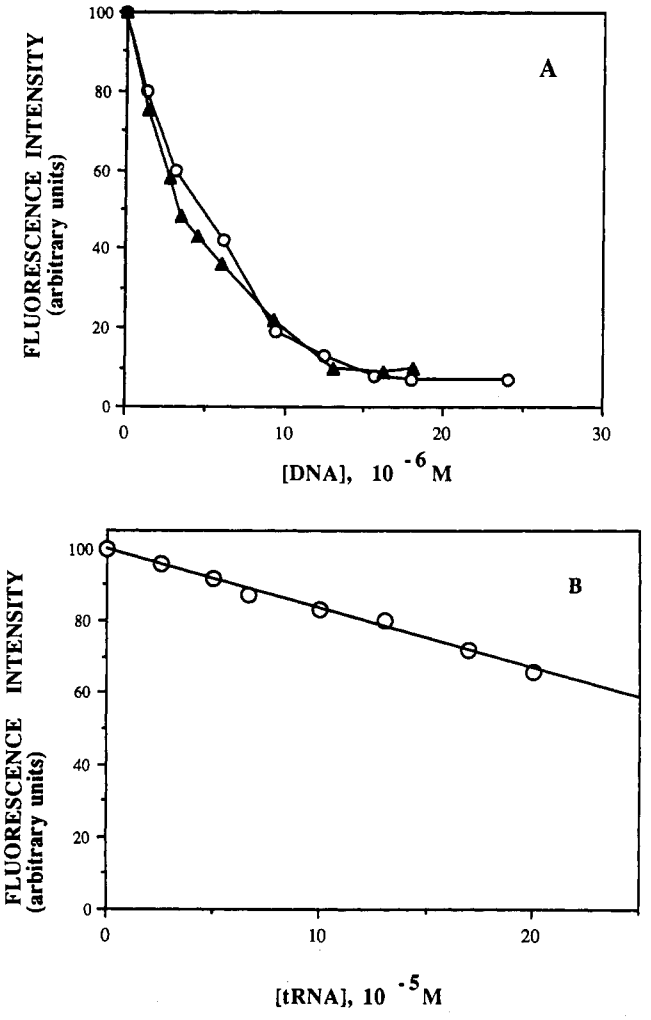

FIGURE 1: Titration of adriamycin with single-stranded DNA of phage M13 and double-stranded DNA of Bluescript plasmid (A) and tRNA (B) monitored by fluorescence quenching. The samples contained 1.7 μM adriamycin and increasing concentrations of nucleic acids, in 10 mM sodium phosphate buffer, pH = 7.3, [NaCl] = 50 mM: ○, double-stranded DNA; △, single-stranded DNA.

As shown in Figure 2, the rate of hydroxyl radicals formation and the maximum concentration of the spin adduct DMPO-OH are only marginally affected by the presence of nucleic acids in the solution but do depend on the presence of an $Fe(EDTA)_2$ complex. The addition of catalase (200 U/mL) totally inhibited Adr-mediated hydroxyl radical generation, whereas superoxide dismutase (30 μg/mL) had only a negligible effect (not shown). Formation of the spin adduct was not detected in the absence of either the drug (Figure 2) or the reductase (not shown). Therefore, neither DNA nor RNA influence Adr-dependent production of oxygen radicals.

DNA and RNA Cleavage. Adr-mediated cleavage of single- and double-stranded DNA and RNA in the presence of NADPH, reductase, and Fe^{3+}–EDTA was examined and is depicted in Figure 3. ds-DNA and ss-DNA undergo strong degradation in the presence of Adr (lanes c and f). In the absence of Adr (lanes d and g), reductase, and NADPH (data not shown) neither ds-DNA nor ss-DNA was degraded. Without the $Fe(EDTA)_2$ complex, both ds-DNA and ss-DNA were degraded to a much lesser extent. Addition of catalase fully inhibited degradation of ds-DNA (Figure 3, lane e). While DNA was readily cleaved by Adr, no degradation of ss-RNA (lane a) or ds-RNA (not shown) occurred. In experiments shown in Figure 3, the concentration of RNA used was lower than that of DNA (see Materials and Methods),

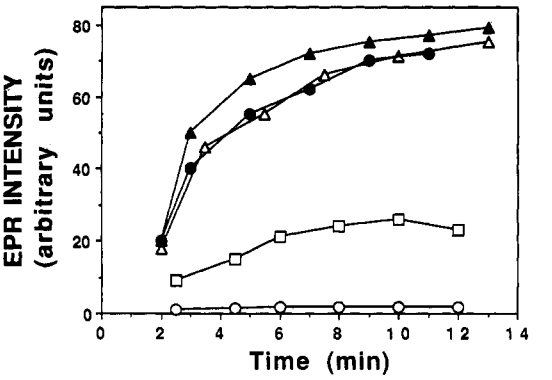

FIGURE 2: Kinetics of the formation DMPO-OH spin adduct in the system containing 100 μM adriamycin, 20 μM $Fe(EDTA)_2$, 2 mM NADPH, and 0.2 unit/mL of NADPH-cytochrome P-450 reductase (▲): ●, in the presence of 100 μM tRNA; △, in the presence of 100 μM DNA; □, in the absence of $Fe(EDTA)_2$; ○, in the absence of adriamycin. The quantitation was based on the height of the second peak of the DMPO–OH EPR spectrum.

although the radioactivity was the same. It should be noted that the same experiments were performed with the end-labeled RNA at concentrations higher than that of DNA (see Materials and Methods). Adr-dependent RNA scission was not observed in this case either (data not shown).

To rule out the possibility that RNA is inherently less susceptible to cleavage by hydroxyl radicals, the same DNA and RNA species were incubated in an artificial hydroxyl radical generating system, normally used in footprinting experiments, consisting of ascorbate–$Fe(EDTA)_2$–H_2O_2 (Tullius & Dombroski, 1986). As shown in Figure 4 (lanes a, b), ss-RNA undergoes significant degradation.

DISCUSSION

We have demonstrated that neither single- nor double-stranded RNA is degraded in aerobic conditions in the presence of Adr, whereas both double- and single-stranded DNA can be effectively cleaved. The sequence of reactions that take place in the presence of Adr and NADPH-cytochrome P450 reductase can be described as follows:

$$\text{Adr} + e \rightarrow \text{Adr}^{\bullet -} \quad (3)$$

$$\text{Adr}^{\bullet -} + O_2 \rightarrow O_2^{\bullet -} + \text{Adr} \quad (4)$$

$$O_2^{\bullet -} + O_2^{\bullet -} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (5)$$

$$\text{Adr}^{\bullet -} + \text{Fe(III)} \rightarrow \text{Adr} + \text{Fe(II)} \quad (6)$$

$$H_2O_2 + \text{Fe(II)} \rightarrow OH^- + OH^{\bullet} + \text{Fe(III)} \quad (7)$$

The •OH radical generated in reaction 7 is assumed to be responsible for nucleic acid cleavage *in vitro* (Imlay & Lihn, 1988). Indeed, catalase, which effectively inhibits the production of •OH radicals, also inhibits Adr-dependent DNA degeneration (Figure 3, lane e). There are three possible explanations why Adr fails to induce free-radical cleavage of RNA: (1) RNA can somehow inhibit the •OH radical production in the above described reaction chain, (2) the chemical structure of RNA, being different from that of DNA, provides protection from free-radical cleavage, and (3) Adr can induce cleavage of nucleic acids only when in complex with it; RNA–Adr complexes cannot be formed. The first explanation is unlikely since we have demonstrated in spin–

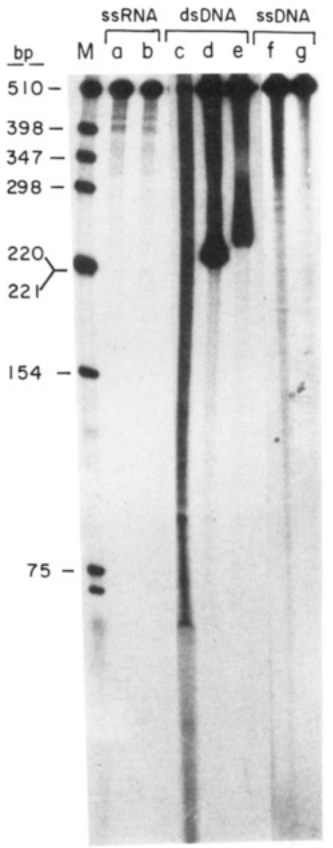

FIGURE 3: Cleavage of radioactively labeled double-stranded and single-stranded DNA and single-stranded RNA by adriamycin. Lanes b, d, and g: nucleic acids in the absence of adriamycin. Lanes a, c, and f: nucleic acids degradation induced by adriamycin. Lane e: the suppression of Adr-dependent DNA cleavage by catalase (300 units/mL). Experimental details are in the text.

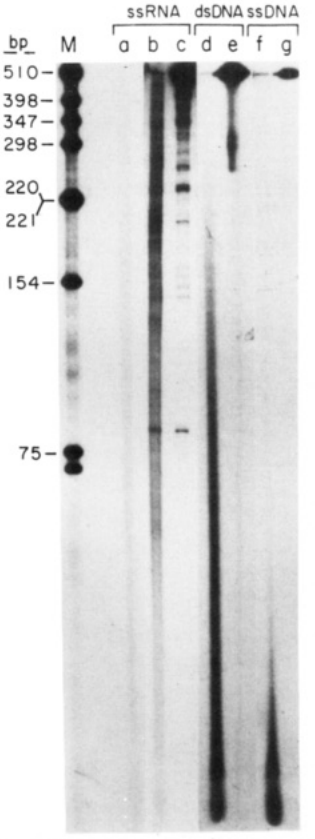

FIGURE 4: Nucleic acid degradation in ascorbate–Fe(EDTA)$_2$–H_2O_2-dependent reaction ("chemical" footprinting). Lanes c, e, and g: untreated control nucleic acids. Lanes a, d, and f: induced nucleic acids degradation. Experimental details are in the text. Experiments shown in lanes a, c, d, e, and f were carried out at 37 °C. RNAs shown in lane b was incubated at 22 °C.

trap experiments (Figure 2) that the rate of production and quantity of produced •OH radicals are approximately the same regardless of the type of nucleic acid (DNA or RNA) present in the reaction mixture. The second explanation is also unlikely because, as shown in Figure 4, •OH radicals generated by the "chemical" method (Fenton reaction) easily induced degradation of both DNA and RNA. Degradation of RNA at 37 °C was even more effective than that of DNA (lanes a and d, respectively). Our observation confirms previous data on cleavage of single- and double-stranded DNA and RNA in the footprinting systems (Celander & Cech, 1990; Prigovich & Martin, 1990).

Thus, the third explanation is most consistent with the results presented here. DNA (Figure 1A) caused effective quenching of Adr fluorescence. The calculated Adr–DNA binding constants (K) and the estimated number of binding sites (n) are in agreement with those in the literature, according to which data the binding constants of adriamycin and daunomycin with DNA are ca. 10^6–10^7 M^{-1} and the number of binding sites is 0.2–0.3 (Chaires et al., 1982; Bareello et al., 1988; Chaires, 1990). Most importantly, Adr cannot form strong complexes with RNA (Figure 1B). Note that at an equimolar concentration of DNA or RNA and Adr ($\sim 10^{-4}$ M), 20% of Adr was bound to DNA, while less than 1% of Adr was bound to RNA. Taken together, the data on nucleic acid–Adr binding (Figure 1) and on degradation of nucleic acids after incubation with Adr (Figure 3) allow us to conclude that Adr induces free-radical cleavage of nucleic acid only if complexed with it.

Our data (Figure 2) suggest that the cascade of reactions described above, induced by Adr and leading to the generation of •OH radicals, takes place regardless of the presence of nucleic acids. We suggest that the sixth reaction (see above) can occur both in solution, when the semiquinone of Adr is free, and on the surface of the nucleic acid, when the semiquinone of Adr is complexed with it. In the latter case, the reduction of H_2O_2 and the formation of •OH radicals would occur on the DNA surface. We propose that only •OH radicals generated in close proximity to the target nucleic acid are responsible for the cleavage induction. The principle ability of semiquinones (e.g., of daunomycin) to interact with DNA has recently been demonstrated (Houee-Levin et al., 1991). Thus, our results prove the dependence of nucleic acid degradation on *in situ* free-radical production by Adr. The importance of •OH radical generation at the site of their action has long been discussed in conjunction with the Fenton method of chemical DNA footprinting. It was proposed that the nucleic acid cleavage is induced by complex formation between DNA and metal ions (Fe, Cu) and by generation of •OH radicals *in situ* (Samuni et al., 1983; Halliwell, 1987).

It remains unclear why the •OH radicals chemically generated in solution (as in the Fenton footprinting system) caused RNA degradation, while the same radicals generated in solution in an Adr-dependent reaction did not. The crucial difference between these two systems is the steady-state level of •OH radicals generated. In the case of "chemical" cleavage, the reaction mixture contains high levels of exogenously induced H_2O_2 and Fe(II) (see Methods), so the resulting concentration gradient of •OH radicals in solution is probably sufficient to induce RNA scission. For the Adr-dependent reaction (see reactions 3–7), the concentration of •OH radicals generated in reaction 7 is limited by the concentrations of

molecular oxygen in solution (~0.25 mM) and Fe(II), produced in reaction 6. Despite the high rate constant of this reaction (Butler et al., 1985), its yield can be low because of a low steady-state level of Adr semiquinone radicals. When Adr–semiquinone is complexed with DNA, •OH radicals formed in proximity to the nucleic acid are at sufficiently high local concentration and, therefore, can cause effective DNA scission. When the semiquinone of Adr is free, as in the case of RNA, the •OH radicals formed in solution do not reach the nucleic acid and are, probably, trapped by low molecular weight components of the system (e.g., NADPH), preventing RNA cleavage.

Our experimental data are explained well in eqs 3–7, assuming that •OH radicals are the main species responsible for DNA scission. At the same time, it is known that in the course of reduction adriamycin is chemically activated and can bind covalently with DNA (Sinha & Chighell, 1979) and cause DNA–protein crosslinks in cells (Potmesil et al., 1983). We cannot completely rule out the possibility of DNA damage as a result of reductive activation of Adr itself, although incubation of DNA with semiquinone radicals of Adr or reduction of Adr under anaerobic conditions by NADPH-cytochrome P-450 reductase did not lead to any notable degradation of DNA (Berlin & Haseltine, 1981).

What is the reason for the inability of Adr to form stable complexes with RNA, whereas such complexes are readily formed with DNA? This cause must be independent of the nucleotide sequence of the molecules, since all radiolabeled nucleic acid molecules used in our footprinting experiments had the same nucleotide sequence (the same plasmid served as a source of both DNA and RNA). The differential binding is probably not due to the strand composition of nucleic acids: both ss- and ds-DNA could bind Adr (Figure 1A) and were readily cleaved (Figure 3, lanes f and c, respectively), while both ss- and ds-RNA could not bind Adr (Figure 1B) and were not cleaved (Figure 3, lane a and not shown, respectively). (Binding experiments were not done with ds-RNA, still, tRNA is known to contain several double-stranded stretches.) It is possible that two parameters that distinguish DNA from RNA can contribute to the selectiveness of Adr binding: sugar composition (desoxyribose in DNA versus ribose RNA) and secondary structure (B-form spiral in DNA versus A-form spiral in RNA). In this context, we note that anthracycline antibiotics are known to form stable complexes with nucleic acids in the B-conformation and hardly react with nucleic acids in the A-conformation (Doskocil & Fric, 1973; Plumbridge & Brown, 1979).

X-ray (Frederick et al., 1990) and NMR (Dinh et al., 1984) analyses suggest that Adr interacts with DNA by intercalation between the bases of a double-stranded molecule. The question arises as to the nature of the complex between Adr and ss-DNA. It is possible that Adr intercalates with the double-stranded stems formed in single-stranded DNA. In fact, the single-stranded DNA of the phage M13, which was used for quenching Adr fluorescence, can form several double-stranded structures, e.g., 19 bp stem at position 5650, 13 bp stem at 6188, or 11 bp stem at 6472. The latter stem is composed predominantly of G–C pairs that are preferential sites for Adr intercalation (Culliname & Phillips, 1990). These and other short hairpins are potential Adr binding sites.

Correlation between the biological (therapeutic) activity of anthracycline antibiotics and their affinity to DNA has long been a matter of interest. It was previously found that methylation of hydroxyl groups at positions 6, 9, and 11 of Adr dramatically decreased its affinity to calf thymus DNA and reduced the drug's antitumor activity (Zunino et al., 1981; Quadrifoglio et al., 1982). The investigators considered their finding to constitute proof for the importance of intercalation of antibiotics with DNA and as evidence against a mechanism implying free-radical cleavage of nucleic acid, because both methylated and nonmethylated derivatives of the drug possessed the same redox properties. In our opinion, their work is in very good agreement with our hypothesis, which links the two mechanisms proposed for Adr action together and proves the requirement for formation of drug–DNA complexes for the free-radical-induced nucleic acid cleavage.

It is known that Adr can localize not only in the nuclei but in other cellular compartments as well. Accordingly, its reduction and formation of $O_2^{\bullet-}$ and H_2O_2 can take place at any location within the cell. Still, we assume that reduction Fe(III) to Fe(II) and subsequent Fenton reaction (reactions 6 and 7) can take place only near the DNA surface. Taking into account the capability of $O_2^{\bullet-}$ radicals and H_2O_2 to penetrate across biological membranes (Lynch & Fridovich, 1978; Rumyantseva et al., 1979; Halliwell & Gutteridge, 1989), we speculate that $O_2^{\bullet-}$ radicals induced by Adr in various cellular compartments may stimulate DNA cleavage in the nucleus (namely, not in the site of their generation).

Our work raises the possibility of creating site-specific antitumor drugs by targeting them to various sequences in cellular DNA or RNA, known to participate in carcinogenesis. Increasing the drug specificity would probably make it possible to reduce therapeutic doses and thus decrease side effects.

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