Does Adriamycin Induce Interstrand Cross-Links in DNA?†

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ABSTRACT: Under nonenzymatic conditions *in vitro*, Adriamycin appears to form interstrand cross-links with DNA over 1–2 days. This is the first report of such Adriamycin-induced interstrand cross-links *in vitro*. Cross-links were measured by a fluorescence based renaturation assay and also by gel electrophoresis. Both procedures revealed an increase of cross-linking with reaction time and with increasing Adriamycin concentration and a 5–6-fold enhancement in the presence of Fe^{3+} ions. The cross-link contains the Adriamycin chromophore, with a λ_{max} of 508 nm, intercalated at the GpC site of cross-linking. Maximal stoichiometry of the cross-link was one per 11–20 bp. The cross-link appears to involve adducts of the Adriamycin chromophore linked to the N^2 of guanine, with no indication that N^7 of guanine is involved. Given that the mode of action of Adriamycin still remains obscure, even after 20 years of clinical use, the possibility that interstrand DNA cross-links may be associated with the clinical mechanism of action of this drug should now be fully addressed.

Adriamycin (trade name, and also known as doxorubicin, Figure 1) has been in widespread clinical use as an effective anticancer agent for 25 years (Jones, 1982; De Vita, 1989; Chabner, 1992). Although it remains one of the most effective components of combination therapy for the treatment of a wide range of tumors, the mechanism of action of this drug has not yet been resolved. While there is good evidence to support several proposed modes of action involving DNA related damage to template function, impairment of topoisomerase II activity, membrane-related effects, or products of bioreductive activation (Powis, 1987; Myers et al., 1988; Cummings et al., 1991a; Bartoszek & Wolf, 1992; Vichi et al., 1992), no single mechanism accounts for all of the effects associated with the chemotherapeutic use of Adriamycin. The lack of a clearly defined mode of action of Adriamycin has been a major obstacle in the search for new, improved derivatives. Over 600 derivatives have been isolated or synthesized (Acamone & Penco, 1992), and over 500 have been submitted to the NCI for screening (Myers et al., 1988; Arcamone & Penco, 1988; Sinha et al., 1989) with only limited success from this enormous effort (Myers et al., 1988; Arcamone & Penco, 1988; Sinha et al., 1989).

There is a considerable body of evidence to show that an important mode of action of Adriamycin is at the DNA level. Several studies have demonstrated increasing drug activity (over three orders of magnitude) with increasing DNA binding or damage and impairment to DNA template activity (Schwartz, 1983; Valentini et al., 1985). There have also been many demonstrations of other DNA related effects such as impairment to topoisomerase II activity (Potmesil, 1988). Furthermore, in living cell nuclei, single cell fluorescence studies have shown that more than 99.8% of Adriamycin is bound to DNA (Gigli et al., 1988), and in human tumor biopsies over 80% of Adriamycin is associated with DNA (Cummings et al., 1986).

From the time that bioreductive alkylation of DNA by anthracyclines was first proposed (Moore, 1977), there have been many attempts to confirm this proposal. Sinha (Sinha & Chignell, 1979; Sinha, 1980; Sinha & Gregory, 1981) showed that Adriamycin-induced adducts could indeed be formed by vigorous chemical reduction under anaerobic conditions, but it was not until recently that an anthracycline quinone methide (the reduced form of anthracyclines, Figure 1) was shown to bind covalently to a nucleophilic site of a nucleic acid component (Egholm & Koch, 1989). There have also been a number of reports of Adriamycin–DNA adducts formed by enzymatic (Bartoszek & Wolf, 1992; Pan et al., 1980; Sinha et al., 1984; Fisher et al., 1983, 1985; Cummings et al., 1991b), microsomal (Sinha & Gregory, 1981), or cellular activation of the drug (Bartoszek & Wolf, 1992; Konopa, 1990; Sinha & Sik, 1980), and this topic has recently been reviewed (Cummings et al., 1991a; Phillips et al., 1990).

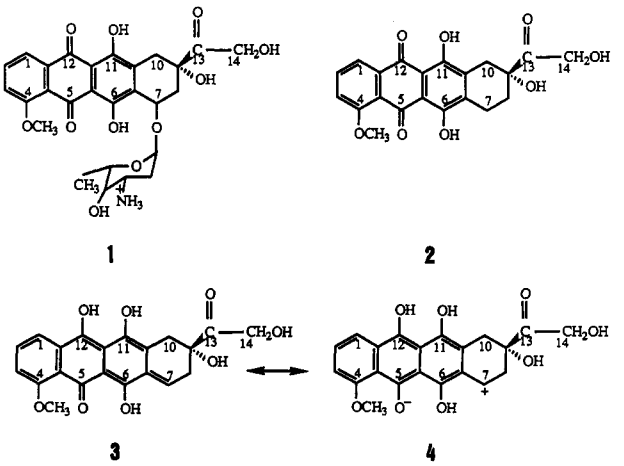

FIGURE 1: Structure of Adriamycin (**1**), 7-deoxyadriamycinone (**2**), Adriamycin quinone methide (**3**), and its tautomer, C7 carbocation of Adriamycin (**4**).

In the process of our *in vitro* transcriptional studies of reversible Adriamycin–DNA interactions, we noted that additional drug blockage sites resulted after long exposure of Adriamycin to the initiated transcription complex (Phillips et al., 1989). These additional drug sites were distinct from intercalation of the drug and could be readily resolved by their different sequence specificity (blockages at G of GpC sites) and the fact that they were essentially irreversible

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(Phillips et al., 1989). Further analysis by bidirectional transcription footprinting revealed that the irreversible unit was restricted to the 2 bp element GpC, and that the transcriptional blockages were enhanced in intensity 10–20-fold by the presence of Fe^{3+} ions (Cullinane & Phillips, 1990).

Since the irreversible transcriptional blockages detected *in vitro* displayed the characteristics of covalent adducts, we sought to characterise these adducts in order to enable further studies to be undertaken to test the proposal that such adducts may be an important mechanism of action of Adriamycin *in vivo*. We present here the first evidence that these adducts, formed by activation of Adriamycin under non-enzymatic reducing conditions *in vitro*, comprise interstrand cross-links in DNA, and that the cross-link contains the Adriamycin chromophore.

MATERIALS AND METHODS

Adriamycin. Adriamycin hydrochloride was supplied by Farmitalia Carlo Erba, Milan. [14-^{14}C]Adriamycin was purchased from Amersham, U.K.

DNA. Calf thymus DNA was from Worthington Biochemical Corp., Freehold, NJ, while the synthetic alternating copolymers poly(dG-dC) and poly(dI-dC) were from Pharmacia, Sweden. The plasmid pSP65 was grown in *Escherichia coli* JM101 cells and isolated using standard methods (Sambrook et al., 1989). For reactions with [^{14}C]Adriamycin pSP65 was linearized with *Pvu*II.

For gel cross-linking studies pSP65 was linearized with *Eco*RI prior to 3′ end-labeling by Klenow fragment of DNA polymerase I in the presence of [α-^{32}P]dATP (Sambrook et al., 1989). Unincorporated label and protein was removed by passing the labeling mixture through a Nensorb20 column (DuPont NEN Products, Boston, MA). The DNA was eluted, lyophilized, and resuspended in sonicated calf thymus DNA in TE[1] buffer to a final concentration of 300 μM bp.

The DNA fragments containing the modified dGTP residues (C^7 or N^7 derivatives) were prepared by DNA thermal cycling. Fifty nanograms of a 497 bp template used previously in transcription studies (Cullinane & Phillips, 1990) was amplified in a buffer containing 10 mM Tris, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.01% Tween 20, and 0.01% NP40, 1 μM each of two 20-mer oligonucleotide primers, 200 μM each of dATP, dCTP, dTTP, and either N^7-dGTP or C^7-dGTP and 2.5 units of Taq DNA polymerase (IBI) in a 100-μL reaction volume. Thermal cycling was performed using a DNA thermocycler (Perkin-Elmer Cetus) programmed at 94 °C for 1 min, 56 °C for 2 min, and 72 °C for 1 min, for 30 cycles. A 72 °C, 10-min step was included at the end of the final cycle. The 490 bp amplified fragment was purified from a 1% low melting temperature agarose gel using standard methods (Sambrook et al., 1989).

Formation of Cross-linked DNA. The reaction mixture typically comprised 25 μM bp DNA [calf thymus, end-labeled pSP65, amplified DNA or the synthetic copolymers poly(dG-dC) or poly(dI-dC)], 10 μM Adriamycin, and 20 μM $FeCl_3$ in transcription buffer (40 mM Tris, pH 8.0, 100 mM KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, and 2–7 mM DTT) (Cullinane & Phillips, 1990). The lower level of DTT was adequate to produce maximal amounts of interstrand cross-links, but higher levels were required to maximize the extent of formation of drug-induced adducts. Reaction volumes were 10–30 μL for gel electrophoresis and [^{14}C]Adriamycin studies, 750 μL for fluorescence renaturation assay preparations, 20 mL for quantitation of drug chromophore by visible absorbance, and 90 mL for atomic absorption determinations. The reaction was normally continued for 48 h at 37 °C. Complete removal of intercalated and unreacted drug was achieved by two phenol extractions followed by one chloroform extraction.

For gel electrophoresis and [^{14}C]Adriamycin studies the DNA was precipitated with ethanol and redissolved in 10–30 μL of TE buffer. For visible absorbance studies of the drug chromophore and atomic absorption studies the DNA was precipitated with ethanol and redissolved in 1.5 and 2.0 mL of transcription buffer, respectively.

Fluorescence Renaturation Assay. The assay for interstrand cross-linking was based on that of Lown et al. (1976). Briefly, 100 μL of 25 μM bp reacted DNA (phenol extracted) was diluted to 1 mL with Milli-Q H_2O (Millipore, Bedford, MA) and taken up to 4 mL with alkaline assay solution (20 mM potassium phosphate, 0.4 mM EDTA, 0.5 μg/mL ethidium bromide, pH 12.0) and divided into two equal aliquots. The fluorescence was measured for one sample (Perkin-Elmer model SPF4 spectrofluorimeter, excitation wavelength 525 ± 5 nm, emission wavelength 600 ± 20 nm) while the other was heat denatured at 70 °C for 5 min and then equilibrated at 20 °C for 5 min prior to measurement of fluorescence. A control sample of DNA, not subjected to Adriamycin, but extracted with phenol, was treated in a similar manner. The percentage of DNA cross-linked was calculated from the expression

$$\% \text{ DNA cross-linked} = \frac{F_t - F_n}{1 - F_n} \times 100$$

where F_t and F_n are the fluorescence readings after heat denaturation, divided by the fluorescence reading before heat treatment, for the drug treated (t) and non-treated (n) DNA. The % cross-linked DNA calculated by this procedure represents the relative number of DNA molecules in this form.

Cross-Linking Gels. The method employed to determine the extent of cross-linking of DNA was based on a gel electrophoresis procedure developed recently (Hartley et al., 1991). End-labeled DNA was reacted with 10 μM Adriamycin at 37 °C under transcription buffer conditions employed previously (Cullinane & Phillips, 1990) both in the absence and presence of 20 μM $FeCl_3$. Aliquots were removed at various time intervals over 48 h, and nonreacted and unbound drug was removed by two phenol and one chloroform extractions. The DNA was subsequently ethanol precipitated and resuspended in 0.2× TE buffer. Denaturing loading buffer was added (to a final concentration of 35% DMSO, 10 mM EDTA, 0.1% xylene cyanol, and 0.1% bromophenol blue) and the DNA denatured at 60 °C for 5 min. The samples were subsequently loaded onto a 0.8% agarose gel and subjected to electrophoresis in TAE buffer at 45 V for 13 h. The gel was dried and autoradiographed and quantitation of band intensities was carried out using a Molecular Dynamics Model 400B PhosphorImager. The percent cross-linked DNA calculated by this procedure reflects the relative number of end-labeled DNA molecules in this form.

Thermal Denaturation. Cross-linked DNA was prepared as described above and redissolved in transcription buffer. The DNA concentration was adjusted to yield A_{260} of 0.5–1.0 and 250 μL degassed with helium for 10 min. Denaturation was carried out with a Gilford model 2527 thermoprogrammer attached to a Gilford 240 spectrophotometer, using a heating rate of 0.5 °C/min.

For thermal lability studies of the cross-link, pSP65 DNA was reacted with [^{14}C]Adriamycin for 48 h as described above. The DNA was subjected to various temperatures for 10 min

[1] Abbreviations: DMS, dimethyl sulfate; DMSO, dimethylsulfoxide; DTT, dithiothreitol; TAE, Tris-acetate-EDTA; TE, Tris-EDTA.

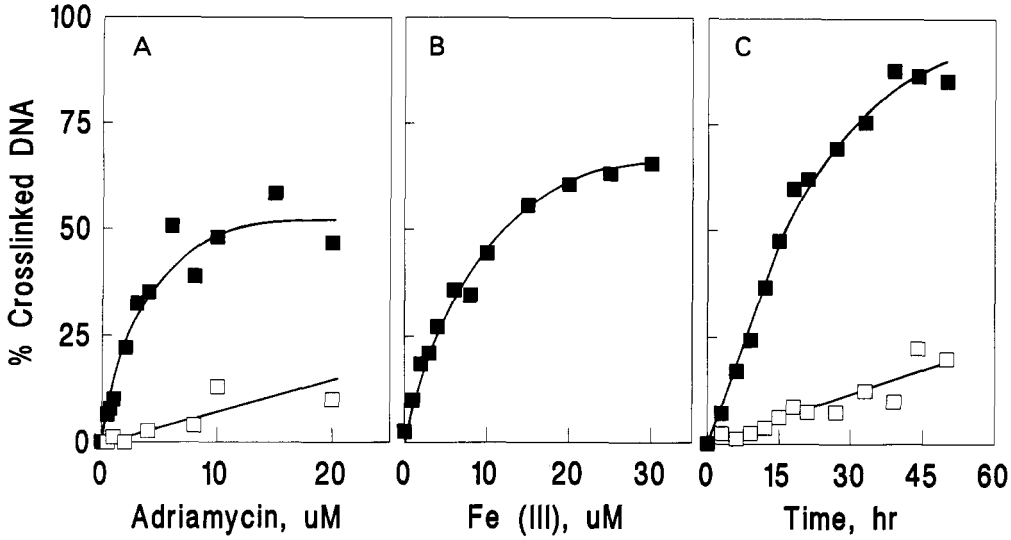

FIGURE 2: Fluorescence renaturation assay of Adriamycin-induced interstrand DNA cross-links. Calf thymus DNA, 25 μM bp, was reacted with Adriamycin in transcription buffer, pH 8.0, in the presence (■) or absence (□) of Fe^{3+} and subjected to phenol extraction. Specific conditions were (A) dependence on Adriamycin concentration (10 μM Fe^{3+}, 24 h reaction time), (B) dependence on Fe^{3+} concentration (10 μM Adriamycin, 24 h reaction time), and (C) dependence on reaction time (10 μM Adriamycin, 10 μM Fe^{3+}).

in transcription buffer. Released drug was removed by phenol extractions and the DNA precipitated with ethanol. The DNA was resuspended in TE buffer and the remaining [^{14}C]-Adriamycin quantitated by scintillation counting.

Spectrophotometry. Ultraviolet and visible spectral measurements were recorded on a Cary 118C spectrophotometer scanning at 1 nm/s. Atomic absorbance measurements were performed on a Varian AA-1475 spectrometer using a Varian FE flow cathode lamp. The instrument was calibrated with a range of standards from 0 to 1.0 ppm, prepared by acid digestion of AR iron wire (Hopkins and Williams, Romford, England) with a lower limit of detection of approximately 0.1 ppm (~0.2 μM).

RESULTS

Interstrand Cross-Links. The apparent Adriamycin–DNA adducts detected previously by an *in vitro* transcription assay resulted from exposure of Adriamycin to an initiated transcription complex under buffer conditions ("transcription buffer", pH 8.0, 7 mM DTT) necessary for subsequent transcription of the DNA (Phillips et al., 1989; Cullinane & Phillips, 1990). In order to establish if these transcriptional blockages were associated with interstrand DNA cross-links, Adriamycin was reacted with DNA under similar buffer conditions. Interstrand DNA cross-links were measured using a fluorescence renaturation assay (Lown et al., 1976) and revealed an increase of interstrand cross-links with increasing drug reaction time, Adriamycin concentration, and Fe^{3+} concentration (Figure 2). After 48 h of reaction time in the presence of Fe^{3+}, cross-linking was enhanced approximately 5-fold compared to the absence of added Fe^{3+}. There was an absolute requirement for DTT, with almost no cross-linking in the absence of this reducing agent and maximal cross-linking at 2 mM DTT (unpublished data).

The fluorescence renaturation assay for detecting interstrand cross-linking is indirect, and it was therefore necessary to confirm such a result by a more direct procedure. For this purpose a sensitive agarose gel electrophoresis cross-linking assay was utilized (Hartley et al., 1991). End-labeled dsDNA (2999 bp) was incubated with Adriamycin for a range of reaction times to induce interstrand cross-links. The DNA was then denatured at 60 °C in 35% DMSO and the resulting single-strand and double-strand DNA resolved by gel electrophoresis (Figure 3A). The intensity of the double-strand DNA band was expressed as a percentage of the total intensity in the two bands in each lane. The inability of DNA to separate into the single-strand form indicates that it is resistant to the denaturing conditions employed, consistent with the presence of an interstrand cross-link. Cross-linking increased in a sigmoidal manner with increasing reaction time and was substantially faster in the presence of Fe^{3+} where 50% of all DNA molecules were cross-linked by 6 h, whereas this level of cross-linking required 40 h in the absence of Fe^{3+} ions (Figure 3B).

Adriamycin Chromophore in the Cross-Link. The detection of Adriamycin-induced cross-links suggested that the Adriamycin chromophore would be involved in the cross-link in some manner. In order to confirm that the Adriamycin chromophore was indeed present, 20 mL of cross-linked DNA was prepared and concentrated to 2 mL. The resultant absorbance spectrum of the cross-linked DNA was similar to that of Adriamycin intercalated into DNA (Figure 4) but exhibited a maxima at 508 nm, compared to 492 nm for intercalated Adriamycin. To confirm that this visible-absorbing species did not arise merely from concentration of residual unreacted Adriamycin remaining after the extraction process, the concentrated DNA–drug adduct was subjected to additional phenol extractions and no loss of visible absorbance was observed.

The rate of formation of the nonextractable form of the Adriamycin chromophore was determined as a function of reaction time (Figure 5A) and was similar to the rate of formation of interstrand cross-links (Figure 2). The rate and extent of formation of adducts was not altered by the absence of EDTA from the buffer (data not shown). After 48 h of reaction time, the chromophore was enhanced 5–6-fold by Fe^{3+}, similar to that detected for the formation of interstrand cross-links (Figure 2). The maximal level of adduct formation reflects a stoichiometry of approximately one chromophore per 20 bp (assuming E_{508} = 5000 M^{-1} cm^{-1} for the adduct), similar to that reported for $NaBH_4$ activation of Adriamycin under anaerobic conditions at the same ionic strength (Sinha, 1980).

Further confirmation that the Adriamycin chromophore was involved in the interstrand cross-link was obtained by the detection of a time-dependent increase of nonextractable [^{14}C]-

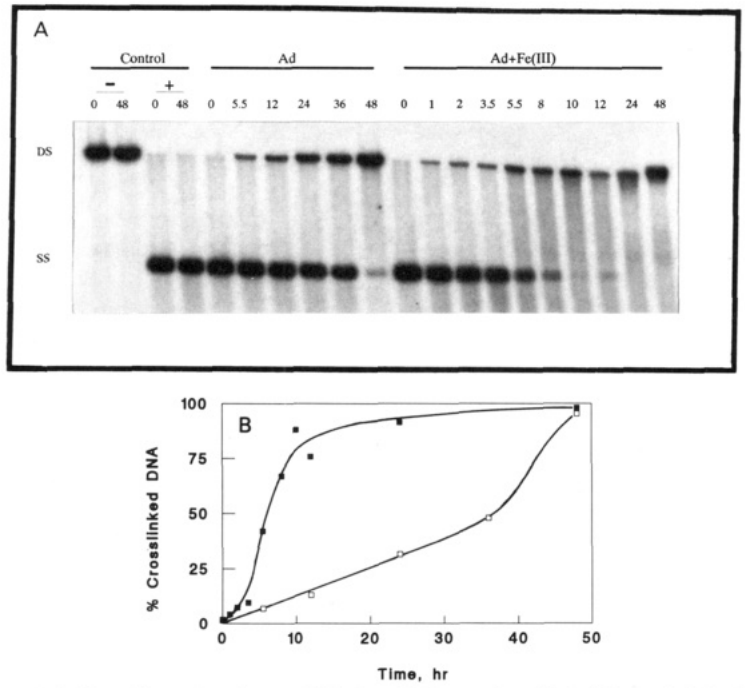

FIGURE 3: Detection of Adriamycin-induced interstrand cross-links by gel electrophoresis. Adriamycin (10 μM) was reacted with end-labeled DNA for 0–48 h in the presence [Ad + Fe (III)] and absence (Ad) of 40 μM Fe^{3+}, phenol extracted and then denatured for 5 min at 60 °C in 35% DMSO prior to electrophoresis. Single-strand DNA is denoted as SS and double-strand DNA as DS. Denatured (+) and nondenatured (–) DNA is shown in control lanes (absence of Adriamycin) at 0 and 48 h reaction times. The autoradiogram is shown in panel A and quantitation of the interstrand cross-link in panel B in the presence (■) and absence (□) of 40 μM Fe^{3+}.

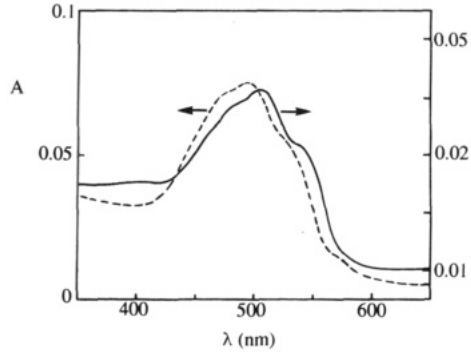

FIGURE 4: Absorbance spectrum of the chromophore associated with Adriamycin-induced cross-linked DNA formed by the reaction of calf thymus DNA (25 μM bp, 48 h) with 10 μM Adriamycin in the presence of 10 μM Fe^{3+} (continuous line). The DNA was extracted with phenol, ethanol precipitated, and redissolved in 2 mL of transcription buffer. For comparison, the intercalated Adriamycin chromophore is also shown in transcription buffer, pH 8.0 (dashed line). The calf thymus DNA concentration was 25 μM bp, the Adriamycin concentration 10 μM, and the reaction was allowed to equilibrate for 5 min.

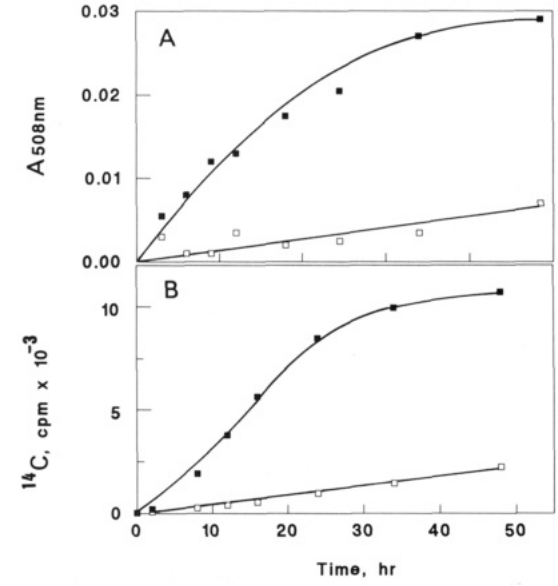

FIGURE 5: Rate of formation of adducts with DNA in the presence (■) and absence (□) of 10 μM Fe^{3+} in transcription buffer, pH 8.0. (Panel A) Calf thymus DNA, 25 μM bp, was reacted with 10 μM Adriamycin and the absorbance of nonextractable chromophore measured at 508 nm. (Panel B) pSP65, 25 μM bp, was reacted with 10 μM [^{14}C]Adriamycin and the nonextractable ^{14}C remaining associated with the DNA measured by liquid scintillation counting.

Adriamycin bound to DNA (Figure 5B). The rate of incorporation of ^{14}C into DNA was identical to that observed by absorbance quantitation (Figure 5A) and interstrand cross-linking (Figure 2). After a reaction time of 48 h the stoichiometry was approximately one [^{14}C]Adriamycin adduct per 11 bp. These stoichiometries are in general accord with that expected from bidirectional transcription footprinting of Adriamycin–DNA adducts where transcriptional blockages were predominantly restricted to just GpC sites and comprise one site per 16 bp. There was virtually no dependence on the extent of formation of either adducts or cross-links on whether the reaction was carried out under aerobic or anaerobic conditions (Table 1).

Thermal Stability and Lability. The possibility remained that the Adriamycin-induced cross-links arose merely from thermal stabilization of the DNA from increasing amount of adducts formed with increasing reaction time. This is not the case since there was no detectable thermal stabilization induced by the adducts—both the control DNA and cross-linked DNA exhibited a melting temperature of 87 °C at 260 nm. However, when absorbance changes were monitored at 508 nm, the apparent melting temperature was 94 °C, with a hyperchromicity of 30%. These values are indicative of release of the drug chromophore from an intercalated state (Porumb, 1978) after much of the DNA had denatured.

The drug chromophore was increasingly extracted by phenol following exposure to heat as low as 50 °C (Figure 6). Complete loss of the chromophore was not accomplished until approximately 90 °C. Extraction of the chromophore was

Table 1: Effect of Oxygen on the Formation of Adducts and Interstrand Cross-Links

reaction conditions	A_{508}[a]	cross-linked DNA (%)[b]
+ oxygen[c]	0.045	63
− oxygen[d]	0.048	69

[a] The absorbance of the purified (phenol extracted) DNA adduct was measured at 508 nm. Adducts were formed as described in the legend to Figure 4. [b] Cross-linked DNA was formed as described under Materials and Methods. [c] Equilibrated with atmospheric level of oxygen. [d] Degassed with nitrogen (10 min).

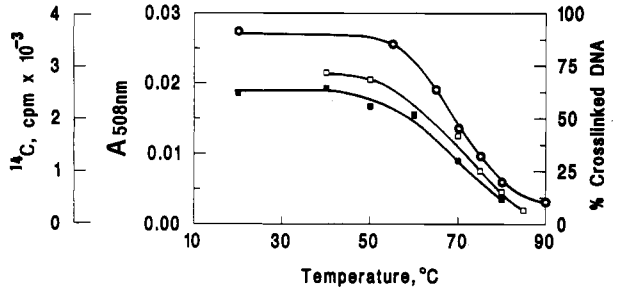

FIGURE 6: Thermal stability and denaturation of cross-linked DNA. Cross-linked DNA (■) was formed with calf thymus DNA as outlined in the legend to Figure 2, phenol extracted, and then subjected to a 10-min exposure to each temperature prior to quantitation of the remaining cross-linked DNA by the fluorescence renaturation assay. Drug–DNA adducts were formed over 48 h as outlined in the legend to Figure 5, phenol extracted, and then subjected to a 10-min exposure prior to quantitation of the remaining ^{14}C (○) or absorbance at 508 nm (□).

monitored both spectrophotometrically and by using [^{14}C]-Adriamycin, with both procedures yielding a midpoint melting temperature of 70 °C. The loss of cross-linking, as measured by fluorescence renaturation assay, completely paralleled the heat-induced loss of adducts and also exhibited a melting temperature of 70 °C (Figure 6).

Absence of Iron. The relatively high affinity of Fe^{3+} for Adriamycin, with an overall stability constant calculated as 10^{16}, 10^{28}, and 10^{33} M^{-3} (Gelvan & Samuni, 1988; Beraldo, 1985; May et al., 1980), suggested that Fe^{3+} might play a role in bridging between two activated Adriamycin moieties, each of which could be bound covalently to each guanine comprising a GpC site. For this reason 2 mL of approximately 20 μM of cross-link was formed from 90 mL of the initial drug–DNA reaction mixture and then analyzed by atomic absorption spectrometry. No iron was detected in the cross-links even though the cross-link was present at a concentration approximately 100 times greater than the detection limit of the spectrometer.

Guanine N^2 Site of Reaction. Since adducts and cross-links are formed at a similar rate (Figures 2 and 5), and adducts are known to form predominantly at GpC sequences (Cullinane & Phillips, 1990), it is likely that the cross-links form at these GpC sites. It is therefore likely that the cross-link involves one or more of the known (N^2, N^3, N^7, or O^6) reactive sites of guanine (Pullman & Pullman, 1981). Binding of Adriamycin to DNA through the N^7 of guanine was precluded on the basis of two results. DNA cross-linked by Adriamycin was treated with DMS under conditions used to induce G-specific cleavage lanes by the Maxam–Gilbert procedure (Maxam & Gilbert, 1980). Since normal G sequences were obtained, Adriamycin adducts do not appear to protect guanine residues from methylation by DMS (Adriamycin adducts themselves did not induce cleavage at guanine residues), indicating that Adriamycin adducts do not bind to the N^7 site of guanine (unpublished data). Further confirmation that

Table 2: Incorporation of [^{14}C]Adriamycin into DNA

DNA	cpm[a] Adriamycin	cpm[a] Adriamycin + Fe(III)
N^7-dGRP derived fragment[b]	1260	4400
C^7-dGTP derived fragment[b]	1220	4300
poly(dG-dC)	1200	8700
poly(dI-dC)	28	20

[a] Incorporation of [^{14}C]Adriamycin into DNA was determined by the addition of a OptiPhase "Hisafe" 3 scintillation cocktail (LKB, Sweden) and subsequent counting in a Wallac 1410 Liquid Scintillation counter. [b] 490 bp fragment derived from amplification of DNA in the presence of N^7-dGTP or C^7-dGTP.

guanine N^7 was not a site of attachment of Adriamycin was demonstrated by the same level of incorporation of [^{14}C]-Adriamycin into a C^7-dGTP derived DNA fragment compared to a N^7-dGTP derived control DNA (Table 2). To examine the possible role of the guanine N^2 site, the incorporation of [^{14}C]Adriamycin was measured for poly(dI-dC) and poly-(dG-dC). No adducts were detected for poly(dI-dC) (Table 2) compared to the control, suggesting that the amino group at N^2 is the site of attachment for the interstrand cross-link.

DISCUSSION

Evidence for Interstrand Cross-Links. The evidence for the formation of interstrand cross-links comes from two assays, both of which detect the presence of two strands of DNA held together under denaturing conditions which would normally result in separation of the strands of duplex DNA. In principle it is possible that a monoadduct could provide sufficient thermal stabilization to local regions of DNA so that this would serve as a nucleation site for renaturation of the DNA. While this possibility cannot be completely discounted, it is difficult to conceive that such a monoadduct could contribute sufficient thermal stabilization to rigorous denaturing conditions such as pH 12, 70 °C, as employed in the fluorescence renaturation assay (or 60 °C in 35% DMSO for the gel assay).

Composition of Cross-Link. The present data do not permit a model of the cross-link to be formulated with any certainty. The major facts are that the drug chromophore and C9 side chain are part of the adduct and cross-link, and the spectral shift is consistent with chemical coupling of the drug to DNA. While it is not possible at this stage to define the nature of the sites of attachment to DNA, it is useful to speculate briefly on the most likely alternatives.

Under the reductive transcription conditions used to generate Adriamycin-induced interstrand cross-links, it is likely that the predominantly active species is the quinone methide (Figure 1), tautomeric with the C7 carbocation (Moore, 1977; Favaudon, 1982). Since the cross-link involves the symmetric sequence GpC, the carbocation is well suited to a reaction with N^7 or N^2 of guanine since both centres are nucleophilic and accessible (Pullman & Pullman, 1981). The N^7 center does not appear to be involved in cross-link formation, whereas the presence of N^2 was essential (Table 2). At this stage the evidence supports the involvement of only guanine N^2 centers and is supported by the demonstration that menogaril (a related anthracycline antitumor agent) is known to form a covalent adduct with N^2 of guanosine following reductive activation of the drug, with the linkage also involving the nucleophilic C7 carbocation of the drug (Egholm & Koch, 1989). The ability of adduct formation at the C7 site of Adriamycin has also been confirmed (by isolation and characterization of these adducts) following reductive activation of the drug (Gaudiano et al., 1990). While such a C7–N^2 bond would be expected to behave as a classical covalent bond, the presence of residual

Fe^{3+} would permit redox cycling of the chromophore and could result in lability of this bond (Moore, 1977; Myers et al., 1988).

An alternative possibility is that the drug chromophore might involve a Schiffs base between the C13 carbonyl of the drug and the N^2 amino of guanine, and the lability of such a bond would not be unexpected. A similar heat lability has previously been reported for anthramycin–guanine N^2 adducts (Hurley et al., 1979).

A further possibility is that the adducts involve an Fe^{3+} chelate between the drug and DNA. Iron is known to bind with high affinity to both the drug chromophore (May et al., 1980) and also to DNA (Netto et al., 1991). Although the use of phenol to extract free and unreacted drug also results in the extraction of most of the Fe^{3+} (unpublished results), the cross-links which are detected could involve this type of attachment to DNA from residual amounts of Fe^{3+} remaining after the extraction process—such low levels of Fe^{3+} may not be detected by atomic absorbance spectrometry. This type of drug–Fe–DNA coordination complex would also be inherently reversible.

The adduct and interstrand cross-link may involve one or more of the potentially labile bonds outlined above. However, since the site(s) of attachment could well involve other or more complex types of attachment, it is not appropriate to speculate further at this time, and clarification of the chemistry involved in adduct and cross-link formation must await additional data on this potentially complex phenomena.

Since the kinetics of formation of adducts (Figure 5) and interstrand cross-links (Figure 2) are identical, this suggests that once an adduct has formed (slowly under these conditions), the cross-linking process then follows at a faster rate, i.e., the rate-limiting step of the cross-linking process is formation of the drug–DNA adduct.

While it would be expected that reduction of Adriamycin would yield the semiquinone, and hence various reactive oxygen radicals (Myers et al., 1988), this does not appear to occur in the present system—there is no evidence of any degradation of DNA arising from such oxygen radicals when using end-labeled DNA in a denaturing gel electrophoresis system designed to test for such degradation (Cullinane & Phillips, 1990), nor is any observed in the present work under denaturing electrophoretic conditions in the cross-linking assay (Figure 3A, 48 h lane in the presence of Fe^{3+}). There is also no loss of intensity of the 48-h band compared to the zero reaction time band, again indicating the absence of significant degradation of the DNA. All of these results suggest that hydroxyl radicals are not involved in the formation of Adriamycin-induced adducts and cross-links under the transcription buffer conditions employed in this work, and this may be due to some degree to the capacity of DTT to scavenge such radicals (Hertzberg & Dervan, 1984). Although oxygen radicals have been shown to be involved in the formation of DNA–protein cross-links (Stadtman, 1993), such oxygen-mediated processes are also absent in the present system, as shown by the lack of dependence of formation of cross-links on the presence or absence of oxygen (Table 1).

Biological Activity and Interstrand Cross-Linking. Does the biological activity of Adriamycin involve interstrand DNA cross-linking? It is not possible to answer this question definitively at this point of time, but given that the interstrand cross-linking potential of Adriamycin has now been demonstrated *in vitro*, its possible role *in vivo* should be assessed. Evidence in support of the occurrence of a reductively activated quinone methide *in vivo* is the detection of 7-deoxyadriamycinone in patients undergoing Adriamycin therapy (Cummings & McArdle, 1986). This metabolite appears to derive from the reaction of the activated species with water (Myers et al., 1988; Cummings et al., 1991a; Kleyer & Koch, 1983). In addition, the quinone methide has a half life of 15 s (Kleyer & Koch, 1983), sufficiently long for reaction with DNA, especially if the activation step takes place in the nucleus where most Adriamycin is known to localize (Gigli et al., 1988; Cummings et al., 1986). The apparent Fe^{3+}-mediated reductive process observed *in vitro* may also occur *in vivo* since it has been shown that Adriamycin can abstract Fe^{3+} from sources such as ferritin (Demant, 1991; Winterbourn, 1991; Myers, 1992). Furthermore, it has recently been shown that the Fe(III)-Adriamycin complex has a more positive reduction potential than Adriamycin and is therefore more amenable to reductive processes than Adriamycin (Dikalov et al., 1992). However, it is more likely that such reductions are carried out by $1e^-$ and $2e^-$ enzymatic catalysis *in vivo*. The induction of DNA cross-linking by such reduction might be expected to be efficient if the catalysis was localized in the nucleus, and there are many examples of such bioreductive enzymes in the nucleus (Cummings et al., 1991a; Pan & Bachur, 1980; Sinha et al., 1984; Doroshow, 1983; Kappus, 1986; Sinha et al., 1989).

Why have these Adriamycin-induced DNA cross-links not been observed more often? Three reasons can be identified. First, the rate of formation may be slow, even *in vivo*—most studies of Adriamycin–DNA interactions have previously been conducted after a few hours of equilibration time, and only trivial levels of cross-links may be formed in that time. Second, the adducts themselves are heat labile and are therefore lost during the heating steps required in many molecular biology protocols used to probe drug–DNA interactions (e.g., PCR, primer-extension assays, etc.)—these procedures therefore fail to identify either the adducts or interstrand cross-links. Finally, the adducts and cross-links are labile above pH 10–11, with almost complete loss after 3 h at pH 12 (data not shown). Crosslinks are therefore unlikely to be detected by alkaline elution procedures where DNA is exposed to pH 12 for many hours (Kohn et al., 1981). Detection and characterization of such adducts and cross-links can only be accomplished if these known destabilizing conditions are avoided, and adducts have indeed been reported recently from MCF-7 cells when using gentle isolation procedures (Bartoszek & Wolf, 1992).

CONCLUSIONS

It is now clear that Adriamycin can form both adducts and apparently also interstrand cross-links under nonenzymatic conditions *in vitro*. The cross-links are readily detected at 1 μM, which is within the physiological range observed for Adriamycin *in vivo*, and it is therefore likely that they would occur at chemotherapeutic levels. The possibility that these cross-links are an important part of the mechanism of action of Adriamycin now warrants further extensive study. Should these adducts be detected *in vivo*, understanding of the molecular basis of the *in vivo* chemistry of this drug will be dramatically altered and will require major changes to the many drug design and development program of Adriamycin currently in progress.

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