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Base Specific and Regioselective Chemical Cross-Linking of Daunorubicin to DNA

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Abstract: The potent anticancer drug daunorubicin binds to DNA by the process of intercalation. Formaldehyde (HCOH) was found to rapidly and efficiently cross-link the drug to DNA in solution in a reaction the rate of which was strongly dependent upon HCOH concentration. The cross-linked drug remains intercalated into DNA, as judged from the results of absorbance, fluorescence, and circular dichroic spectroscopic studies and thermal denaturation studies. Comparative studies using a series of anthracycline derivatives showed that the 3′-NH_2 group on the daunosamine moiety is absolutely required for cross-linking. Comparative studies using synthetic deoxypolynucleotides of defined sequence showed that the N2 amino group of guanine is absolutely required for cross-linking. In restriction enzyme inhibition assays using pBR322 DNA as a substrate, cross-linked daunorubicin was found to completely inhibit cutting by Nae I (recognition site 5′GCCGGC3′) but not by Dra I (recognition site 5′TTTAAA3′). These results (a) extend, into solution, previous reports of the cross-linking of daunorubicin to oligonucleotides in crystals; (b) show that daunorubicin can be chemically cross-linked to natural DNA samples as well as to poly- and oligonucleotides, and (c) demonstrate the base- and regioselectivity of the cross-linking reaction.

The anthracycline antibiotics, of which daunorubicin (daunomycin) and doxorubicin (Adriamycin) are the parent compounds, are widely used in cancer chemotherapy.[1] After three decades of study, daunorubicin and doxorubicin are arguably the best characterized DNA intercalators.[2] These compounds are important models for understanding how small molecules interact with DNA in a sequence-specific manner.

Recent structural studies reported the serendipitous covalent cross-linking of anthracycline antibiotics to DNA oligonucleotides during crystallization.[3] Trace amounts of HCOH in the precipitant 2-methyl-2,4-pentandiol used for crystallization were found to efficiently cross-link bound drugs to either the N2 group of guanine or the N2 of diaminopurine in oligonucleotides containing those bases in the center of their sequences. The reaction appeared to be facilitated by the proximity of the 3′-NH_2 group in the bound drug to the N2 group of the DNA base

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in the complex, and perhaps by the chemical environment of the DNA minor groove, which has a dielectric constant that differs dramatically from that of the surrounding solvent. Cross-linking to oligonucleotides and to [poly (dGdC)]$_2$ in solution was subsequently reported,[3d] but several aspects of the cross-linking reaction remained unclear. Whether or not anthracyclines can be cross-linked to natural DNA sequences in solution has not yet been established, nor has the specificity of the cross-linking reaction been thoroughly investigated. In addition, the effect of changes in the position of the reactive amine group within the anthracycline structure has not been examined, a necessary step required to evaluate the stringency of the cross-linking reaction.

These questions were addressed by the studies described here. Our studies demonstrate that low concentrations of HCOH can efficiently chemically cross-link daunorubicin to natural DNA. Comparative cross-linking studies using polynucleotides of defined sequence and a series of anthracycline antibiotics revealed a pronounced base- and regiospecificity of the cross-linking reaction. There appears to be an absolute requirement for the N2 of guanine and for an NH_2 group at the 3′ position for efficient cross-linking by HCOH. These features of the HCOH cross-linking of anthracyclines to DNA suggest a new pathway for the rational design of covalent DNA binding agents.

Experimental Section

Materials. Daunorubicin, doxorubicin, proflavine, and ethidium bromide were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without further purification. Actinomycin D was purchased from Boehringer Mannheim (Indianapolis, IN). Hoechst 33258 was obtained from Molecular Probes, Inc. (Eugene, OR). Hydroxyrubicin, WP608, and WP711 (3′-epidaunorubicin) were synthesized as previously described.[4] Adriamycinone was prepared by acidic hydrolysis of doxorubicin and was purified by crystallization from methanol. A molar extinction coefficient of 11 500 M^{-1} cm^{-1} at 480 nm was used to determine the concentration of daunorubicin. Deoxypolynucleotides of defined sequence were obtained from Pharmacia Biotech (Piscataway, NJ). Formaldehyde (37% (w/w) solution, certified A.C.S reagent, lot no. 943148) was purchased from Fisher Scientific (Fair Lawn, NJ) and was used without further purification. Plasmid pBR322 DNA was purchased from New England Biolabs. (Beverly, MA). Restriction endonucleases Nae I and Pvu II were obtained from USB (Cleveland, OH), and Dra I was purchased from Promega (Madison, WI).

DNA Preparations. Herring sperm DNA (Boehringer Mannheim, Indianapolis, IN) was dissolved in BPE buffer (6 mM Na_2HPO_4, 2 mM NaH_2PO_4, 1 mM Na_2EDTA, pH 7.1), then sonicated, phenol-extracted, and dialyzed as previously described.[5] Prior to cross-linking experiments, DNA was dialyzed against a sodium borate buffer (10 mM $Na_2B_4O_7$, pH 8.2). A molar extinction coefficient of 13 000 $M(bp)^{-1}$ cm^{-1} at 260 nm was used to determine the concentration of DNA from absorbance measurements.

Cross-linking Experiments. Cross-linking reactions were conducted at 24 °C in solutions containing DNA, daunorubicin, sodium borate buffer, pH 8.2, and 2% (v/v) HCOH (unless otherwise specified). After 30 or 60 min, reactions were stopped either by the addition of sodium dodecyl sulfate (SDS) to a final concentration of 1.33% (v/v) or by phenol extraction using buffer-saturated phenol in a 1:1 volume ratio. In DNA titration studies, a fixed concentration of daunorubicin (26.7 μM) was equilibrated with increasing concentrations of herring sperm DNA prior to the addition of HCOH to initiate the cross-linking reaction.

Visible Absorption, Fluorescence Emission, and Circular Dichroism Spectra. Solutions containing identical concentrations of either free, noncovalently bound, or covalently cross-linked daunorubicin were prepared for comparative spectroscopic studies. The concentration of drug in these samples was determined by absorbance measurements at the isosbestic point, 540 nm, using the extinction coefficient 5100 M^{-1} cm^{-1}. Visible absorbance spectra were recorded in a Cary Model 219 spectrophotometer at room temperature. Fluorescence emission spectra were recorded in a Perkin-Elmer Model 650-40 spectrofluorometer, using a slit width of 10 nm and $\lambda_{ex} = 480$ nm. Circular dichroism spectra were recorded at room temperature on a Jasco Model J500A spectropolarimeter interfaced to and controlled by an IBM PC computer. The molar ellipticity ([θ]) was calculated from the equation $[\theta] = 100\theta/cl$, where θ is the measured ellipticity in degrees, c is the daunorubicin concentration, and l is the path length in centimeters.

Thermal Denaturation Experiments. Thermal denaturation studies were performed in a Cary 219 spectrophotometer connected to a Neslab RTE-100 programmable water bath. Samples in BPE buffer were heated continuously from 20 to 100 °C at a rate of 1 °C/min, while absorbance changes were continuously recorded at 260 nm. Melting profiles were digitized and analyzed using the program FitAll (MTR Software, Toronto, Canada).

Restriction Endonuclease Digestion Experiments. Plasmid pBR322 DNA (30 μg in 50 μL of a solution containing 10 mM Tris-HCl, pH 7.5, 60 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT) was linearized by digestion with 35 units of Pvu II for 24 h at 37 °C. The linear pBR322 DNA was purified by phenol extraction followed by ethanol precipitation. For use in cross-linking studies, the precipitated DNA was then dissolved in sodium borate buffer (10 mM $Na_2B_2O_7$, pH 8.2). Cross-linking reactions contained (in a total volume of 30 μL) 462 μM(bp) plasmid DNA, variable concentrations of daunomycin, and 2% (v/v) HCOH. Reactions were incubated at 24 °C for 60 min and then stopped by phenol extraction. Samples of cross-linked and control DNA (treated as described above but with no drug present) were then digested by restriction endonuclease as follows: 2 μg of DNA per reaction were digested at 37 °C for 24 h by either Nae I or Dra I. For Nae I, the reaction buffer contained 10 mM Tris-HCl (pH 8.2), 20 mM NaCl, 7 mM $MgCl_2$,1 mM DTT. For Dra I, the reaction buffer contained 6 mM Tris-HCl (pH 7.5), 50 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT. Digestion reactions were stopped by the addition of EDTA. DNA samples were electrophoresed on a 14 cm long 1% agarose gel for 5 h and then stained with ethidium bromide.

Results

Covalent Cross-Linking of Daunorubicin to Herring Sperm DNA by HCOH. Figure 1 shows the time course for the covalent cross-linking of daunorubicin to herring sperm DNA by HCOH. Formaldehyde concentration was varied (1%, 0.5%, 0.25% and 0.125% [v/v]), while DNA (350 μM) and daunorubicin (35 μM) concentrations were kept constant. The individual time courses were fit to a single exponential, yielding a pseudo first-order rate constant. The pseudo first-order rate constant was found to depend strongly on the total HCOH concentration, as shown in Figure 2.

Figure 3 shows the results of the HCOH cross-linking titration experiments in which the DNA concentration was systematically varied. Daunorubicin concentrations were fixed at 26.7 μM in these experiments, while the herring sperm DNA concentrations were increased from 0.554 μM to 1 mM. The resulting data produced a sigmoidal curve when the fraction of drug cross-linked was plotted as a function of the logarithm of the DNA concentration, as expected for simple binding behavior. Under the conditions of this experiment, the total drug concentration

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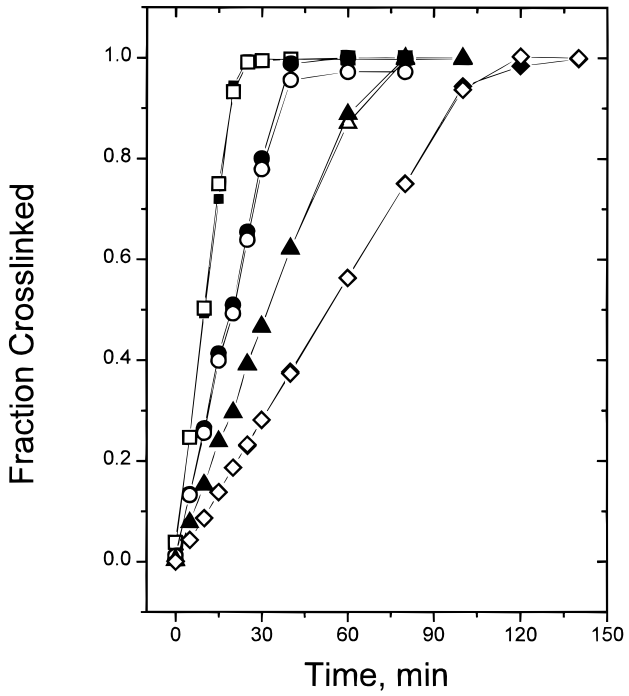

Figure 1. Time course for the covalent cross-linking of daunorubicin to DNA by HCOH. Formaldehyde concentration of 1.0% (v/v) (squares), 0.5% (circles), 0.25% (triangles), and 0.125% (diamonds) were used. The amount of covalently attached drug was determined after extraction by either phenol (solid symbols) or SDS (open symbols). The fraction of drug cross-linked (nonextractable drug/total drug at $t = 0$) is shown as a function time following the addition of formaldehyde.

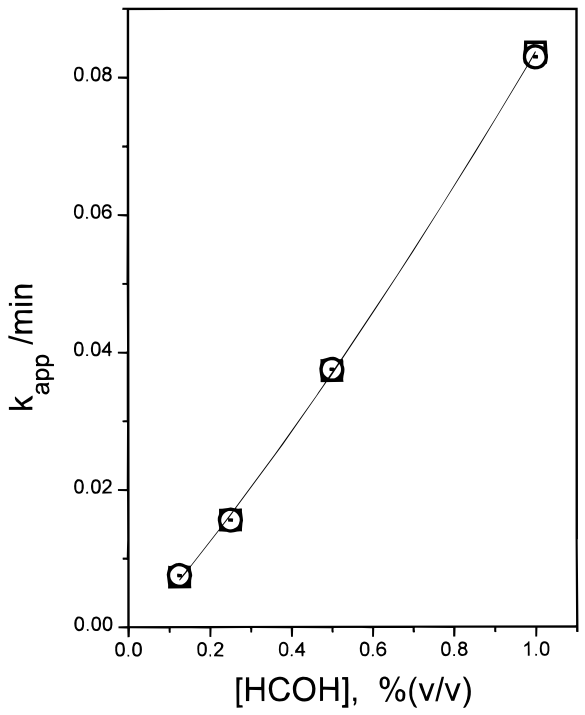

Figure 2. Apparent first-order rate constant for the cross-linking reaction as a function of the HCOH concentration. Data obtained by either the phenol (squares) or SDS (circles) extraction procedures are shown.

is much higher than the reciprocal of its DNA association constant, so its binding is very nearly stoichiometric.

Optical Properties of Free, Bound and Formaldehyde-Cross-Linked Daunorubicin. Figures 4 and 5 show the visible absorbance, fluorescence emission and CD spectra of free,

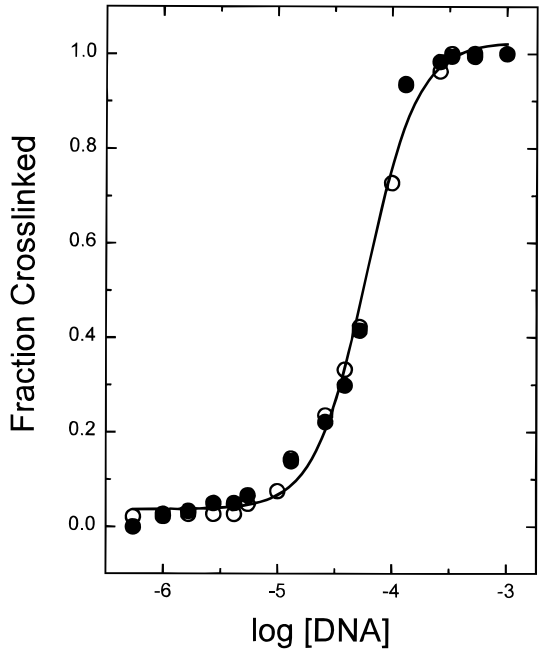

Figure 3. Formaldehyde cross-linking titration experiments. Fixed concentrations of daunorubicin were cross-linked with increasing concentrations of herring sperm DNA by HCOH. Cross-linking reactions were conducted at 24 °C in solutions containing 26.7 μM daunorubicin, 10 μM sodium borate buffer, pH 8.2, and 2% (v/v) HCOH. After 60 min, reactions were stopped by phenol extraction. Open and closed circles refer to separate titration experiments. The fraction of drug cross-linked (F/F_0) is shown as a function of the logarithm of the total DNA concentration.

bound, and HCOH cross-linked daunorubicin. The spectra of HCOH-cross-linked daunorubicin were similar to those of noncovalently bound daunorubicin. In both cases, a red shift of the absorbance maximum from 480 to 505 nm occurred, and the molar extinction decreased (Figure 4). Daunorubicin fluorescence emission was also nearly completely quenched for both cross-linked and noncovalently bound daunorubicin, but the extent of quenching was slightly less in the former case. The CD spectra of noncovalently bound and cross-linked daunorubicin are similar, and both are significantly different from that of the free drug, especially at 300 nm, where a pronounced induced CD band is evident for the bound daunorubicin forms. The induced CD band is consistent with intercalation into DNA for both the cross-linked and noncovalently bound forms.

T_m is Dramatically Increased by Covalently Cross-Linked Daunorubicin. The thermal denaturation of herring sperm DNA alone and in the presence of either noncovalently bound or covalently cross-linked daunorubicin was examined. Differential DNA melting curves are shown in Figure 6. Under these conditions, herring sperm DNA showed a T_m of 60 °C. Both noncovalently bound and covalently cross-linked daunorubicin stabilized the DNA. At a binding ratio of 0.1 mol daunorubicin per DNA base, the T_m is increased by 16 °C for the noncovalently bound form and by 20 °C for the cross-linked form at the same binding ratio. In addition, the melting curve was distinctly biphasic for the noncovalently bound drug but less so for the cross-linked drug.

The Daunorubicin−DNA Cross-Link Is Thermolabile. A limited number of experiments were done to qualitatively examine the heat stability of the cross-linked adduct. Samples of daunorubicin cross-linked to herring sperm DNA were prepared, and the formation of the covalent adduct verified by phenol extraction at 20 °C. Samples were then heated at 96

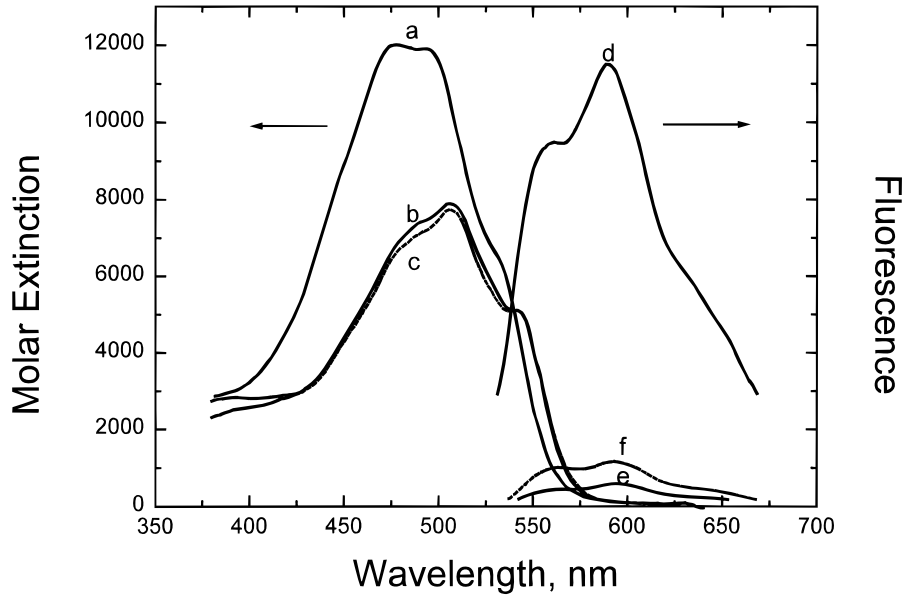

Figure 4. Visible absorption (left) and fluorescence emission (right) spectra of free, bound, and HCOH cross-linked daunorubicin in BPE buffer. Visible absorption spectra: (a) free, (b) bound, and (c) cross-linked daunorubicin. Fluorescence emission spectra (λ_{ex} = 480 nm): (d) free, (e) bound, and (f) HCOH cross-linked daunorubicin.

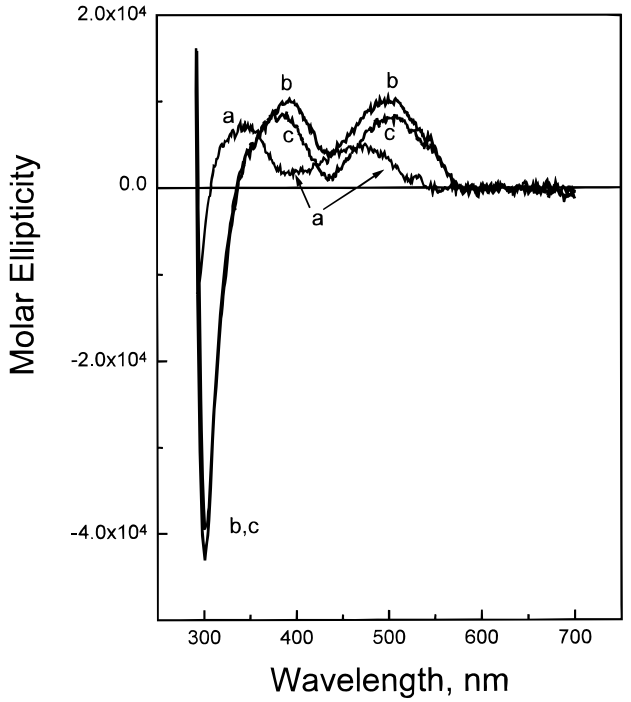

Figure 5. CD spectra of free, bound and HCOH cross-linked daunorubicin in BPE buffer: (a) free, (b) bound, and (c) cross-linked daunorubicin.

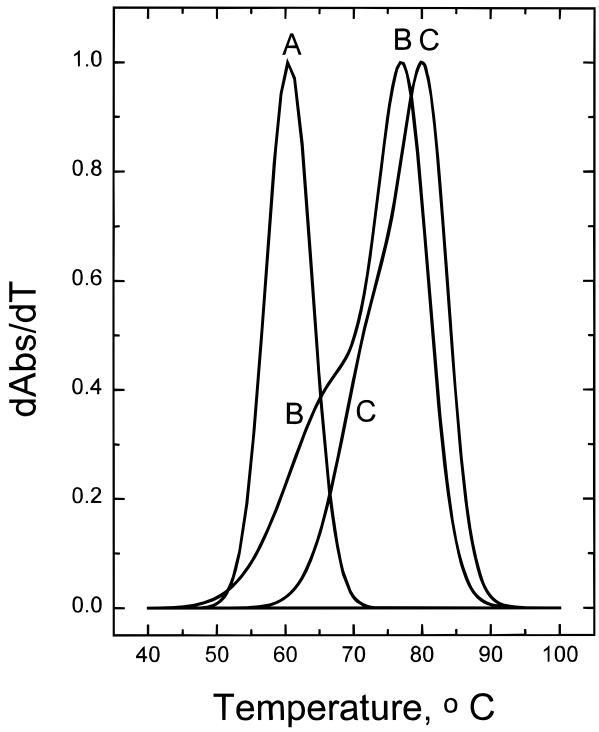

Figure 6. Differential DNA melting curves obtained in the presence or absence of daunorubicin: (A) no daunorubicin, (B) noncovalently bound daunorubicin, and (C) cross-linked daunorubicin.

°C for various lengths of time, cooled to ambient temperature, and phenol extracted. The amount of drug remaining in the aqueous phase, and presumed to be covalently cross-linked, was then determined by visible absorbance measurements. The results of such experiments showed that the drug−DNA cross-link is heat labile and that heating at 96 °C renders the drug susceptible to phenol extraction. Detailed kinetic studies have not been done, but indications are that incubation for 10−20 min at 96 °C renders 50% of the originally cross-linked drug susceptible to phenol extraction.

The 3′-Amino Group Is Essential for Formaldehyde Cross-Linking of Daunorubicin to DNA. Cross-linking to DNA was attempted using several other anthracycline antibiotics (Table 1; Figure 7). Compounds having the 3′-amino group (daunorubicin, doxorubicin, and WP711) could be efficiently cross-linked to DNA. In contrast, those lacking the 3′-amino group (hydroxyrubicin, adriamycinone, and WP608) could not be cross-linked to DNA by HCOH under the same conditions. Ethidium bromide, proflavine, actinomycin D, and Hoechst 33258 could not be cross-linked to DNA under the same conditions used to cross-link anthracycline antibiotics (data not shown).

Formaldehyde Cross-Linking of 3′-NH_2 Anthracyclines to DNA Requires the N2 Amine of Guanine. Deoxypolynucleotides of defined sequence were used to examine the preferred DNA cross-linking sites for daunorubicin (Table 2). Daunorubicin was efficiently cross-linked to [poly (dGdC)]$_2$, poly dG:

Table 1. Covalent Cross-Linking of Anthracycline Antibiotics to DNA by HCOH[a]

comp	C_b^o μM	C_b^x μM
A. daunorubicin	34.7	34.6
	34.9	*34.9*
B. doxorubicin	35.5	35.4
	35.4	*35.4*
C. WP711	28.3	28.2
	28.0	*26.5*
D. hydroxyrubicin	34.3	0.04
	34.3	*0.00*
E. adriamycinone	35.0	0.05
	35.0	*0.02*
F. WP608	34.2	0.04
	34.2	*0.00*

[a] Cross-linking reactions were conducted at 24 °C in solutions containing 350.7 μM DNA (bp), sodium borate buffer, pH 8.2, and 2% (v/v) HCOH. After 30 min, reactions were stopped by phenol extraction or by the addition of SDS to a final concentration of 1.33% (italicized entry). C_b^o is the concentration of bound antibiotic at the start of the reaction. C_b^x is the concentration of antibiotic remaining bound (and presumably covalently cross-linked) to the DNA after SDS or phenol extraction.

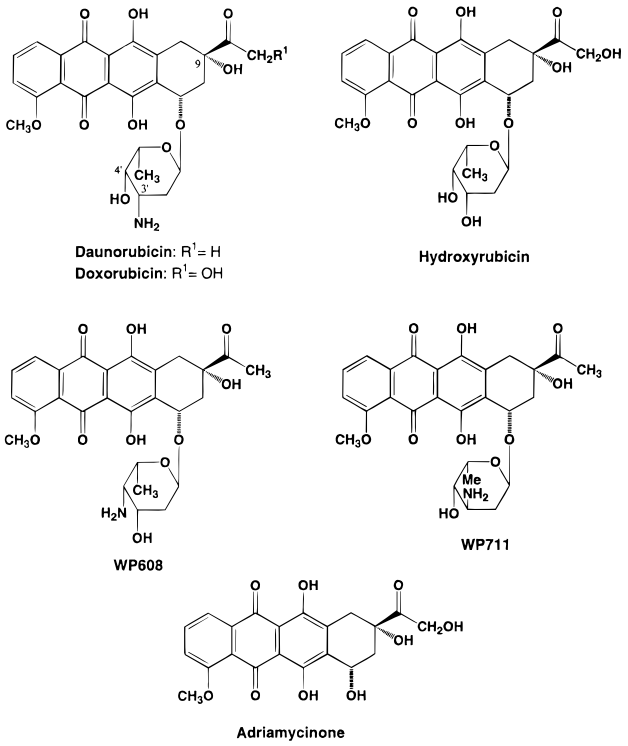

Figure 7. Structures of daunorubicin, doxorubicin, WP711, hydroxyrubicin, WP608, and adriamycinone.

poly dC and poly (dA-dC):poly (dT-dG) but not to [poly (dAdT)]$_2$ or [poly (dIdC)]$_2$. These results indicate that daunorubicin was cross-linked to sequences that contain guanine, suggesting that the N2 amine group of guanine is required for the cross-linking reaction.

Daunorubicin Cross-Linking to Plasmid DNA. Inhibition of restriction endonuclease cleavage of plasmid pBR322 DNA by cross-linked daunorubicin confirmed the guanine specificity of the cross-linking reaction. These experiments used long incubation times, which, in the absence of drug, yielded complete digestion of the plasmid DNA into restriction fragments. The qualitative effect of covalent attachment of drug was examined. Two restriction endonucleases, Nae I (recognition site 5′GCCGGC3′) and Dra I (recognition site 5′TTTAAA3′), were used for this study. Figure 8a shows the results of inhibition of Nae I cutting sites by cross-linking daunorubicin.

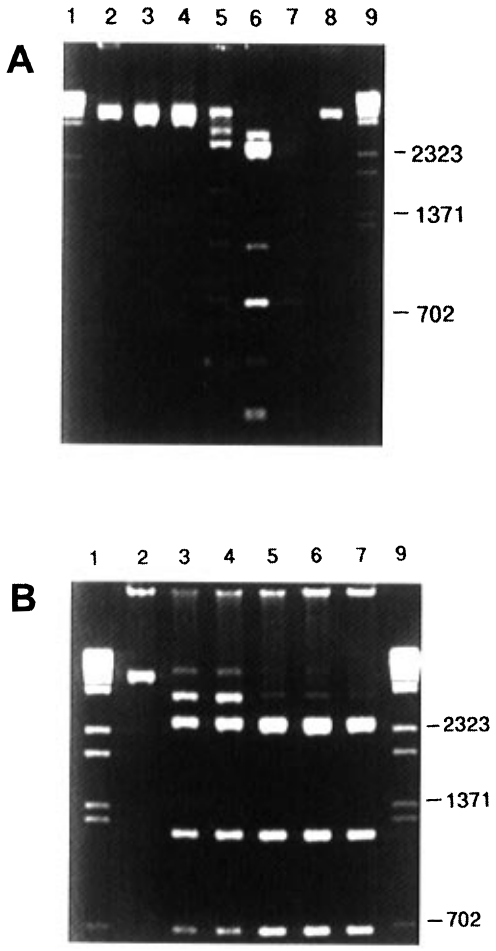

Figure 8. Inhibition of restriction endonucleases Nae I (A) and Dra I (B) cutting sites by HCOH cross-linked daunorubicin. (A) Nae I digestion experiments. Lanes 1 and 9 are size markers, and lane 2 is undigested HCOH-treated pBR322 DNA. Lanes 3−5 are products of the digestion of pBR322 DNA with different concentrations of cross-linked daunorubicin: 175 μM (lane 3), 52.4 μM (lane 4), and 5.24 μM (lane 5). Lane 6 is digested pBR322 DNA which was treated by HCOH. Lane 7 is empty. Lane 8 is undigested, untreated pBR322 DNA. (B) Dra I digestion experiments. Lanes 1 and 9 are size markers, and lane 2 is undigested HCOH treated pBR322 DNA. Lanes 3−5 are products of the digestion of pBR322 DNA with different concentrations of cross-linked daunorubicin: 175 μM (lane 3), 52.4 μM (lane 4), and 5.24 μM (lane 5). Lane 6 is digested pBR322 DNA that was treated by HCOH. Lane 7 is digested pBR322 DNA untreated by HCOH.

Plasmid pBR322 DNA has four Nae I cutting sites, all of which were inhibited by cross-linked drug (Figure 8a, lanes 3−5). High molar ratios of cross-linked drug (Figure 8a, lanes 3 and 4) completely inhibited Nae I cleavage, while low molar ratios of cross-linked drug (Figure 8a, lane 5) only partially inhibited cleavage. Formaldehyde alone showed slight, nonspecific inhibition of Nae I digestion.

Figure 8b shows the results of Dra I inhibition studies. Plasmid pBR322 DNA has three Dra I cutting sites. High molar ratios of cross-linked daunorubicin only partially inhibited cleavage of these sites (Figure 8b, lanes 3 and 4), while lower molar ratios showed no inhibition at all (Figure 8b, lane 5).

These experiments demonstrated that cross-linked daunorubicin completely inhibited cutting by Nae I but minimally affected cleavage by Dra I. Since Nae I has a GC-rich recognition site, while that of Dra I is AT-rich, this finding confirms the guanine specificity inferred from cross-linking studies using polynucleotides. We also used Pvu I (recognition site 5′CGATCG3′) and Hind III (recognition site 5′AAGCTT3′)

in the same type of inhibition studies (data not shown) and found that the efficiency of inhibition of restriction enzyme digestion followed the order: Dra I < Hind III < Pvu I < Nae I (from least to most effective inhibition). This showed that the greater the G+C content of the restriction enzyme recognition site, the greater the extent of inhibition by cross-linked daunorubicin.

Restriction enzyme digestion of pBR322 DNA in the presence of noncovalently bound daunorubicin was also examined. Digestion by Nae I, Pvu I, and Dra I was inhibited, but not that by Hind III. As expected, inhibition by noncovalently bound daunorubicin to DNA was less effective than inhibition by covalently cross-linked daunorubicin.

Discussion

The results presented here extend previous reports of daunorubicin cross-linking to oligonucleotides in the crystalline state. In these studies, we have established that the cross-linking of daunorubicin to DNA by HCOH, in solution, is a reaction both base- and regiospecific. We have defined the structural requirements of both partners of the cross-linking reactions and have proved that the N2 amino group of guanine and the 3′-NH_2 group on the daunosamine are substituents absolutely required for efficient covalent adduct formation.

Mechanism of the Cross-Linking Reaction. Although we did not aim in these studies to establish a detailed reaction mechanism, our data do allow us to sketch minimal elements that must be included in the cross-linking reaction. At least three equilibria must be considered

$$D + S \rightleftarrows C \quad (1)$$

$$C + F \rightleftarrows CF \quad (2)$$

$$CF \rightleftarrows CX \quad (3)$$

where D represents daunorubicin, S is a DNA binding site, C is the drug-DNA complex, F is HCOH, CF is the noncovalent ternary complex containing drug bound to DNA and HCOH, and, finally, CX is the cross-linked ternary complex. This simplest possible set of reactions can qualitatively account for many of the observed aspects of the cross-linking reaction. First, the time scale of the reaction (minutes) is slow, presumably resulting from the rate limiting step 3 in which the cross-link is formed. Daunorubicin binding kinetics have been studied in detail,[6] and the rate of complex formation (step 1) is known to be complete within 1 s. Step 1 will, however, impart a DNA concentration dependence to the cross-linking reaction, as was observed (Figure 3). Similarly, the bimolecular interaction of HCOH with the drug−DNA complex (step 2) would result in an HCOH concentration dependence of the cross-linking rate, as was observed (Figures 1 and 2). Transformation of the data in Figure 2 into a plot of log k_{app} *versus* log [HCOH] yields a straight line, with a slope of 1.16(± 0.1) (not shown). The slope indicates that the cross-linking reaction is essentially first order with respect to HCOH concentration, from which we conclude that stoichiometry of HCOH in the reaction is 1. This conclusion implies that one HCOH molecule is involved in the formation of each cross-linking event, as might be expected. While the detailed reaction mechanism is no doubt more complicated, with more intermediate steps, the equilibria 1−3 provide a reasonable reaction scheme that accounts for the experimental observations.

Other pathways for the cross-linking reaction are, of course, possible. Formaldehyde might, for example, react with the free drug in a bimolecular reaction prior to ternary complex formation. The drug−HCOH complex could then react in a bimolecular reaction with DNA, followed by formation of the cross-link in a unimolecular reaction. Such an alternate pathway is not eliminated by the experimental data now available.

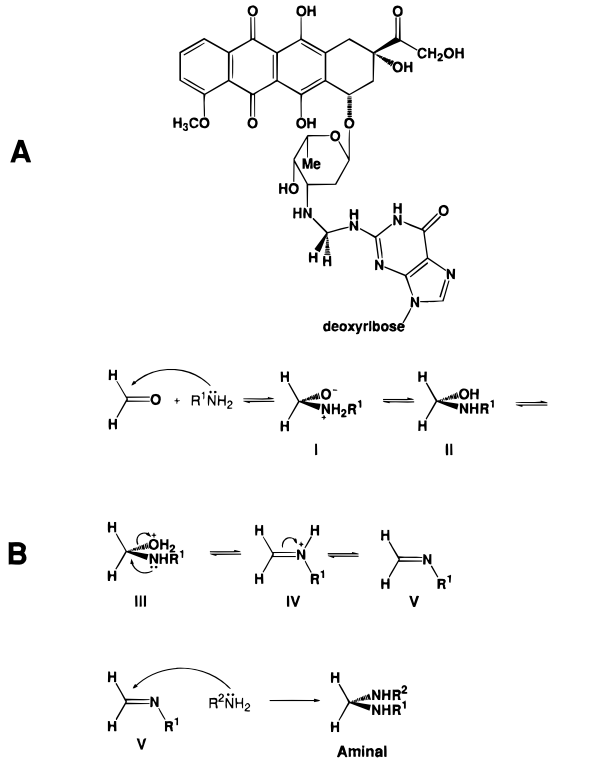

Figure 9. (A) Structure of doxorubicin cross-linked to the N2 group of guanine. (B) A plausible reaction pathway for the cross-linking of the 3′-NH_2 group of daunorubicin or doxorubicin (R^1NH_2) with N2 of guanine (R^2NH_2) by formaldehyde.

The reaction of HCOH with DNA has been extensively studied.[7,8] Formaldehyde reactivity has been used as a probe of the rate of base pair opening to study the dynamics of the DNA double helix.[7] More recently,[8] the mechanism of HCOH cross-linking of the two strands of the DNA double helix has been described. Both of these reactions occur on the time scale of tens of hours to days, several orders of magnitude slower than the cross-linking reactions we describe here. We therefore believe that the reactions studied here are unlikely to be complicated by these other types of reactions because of the large differences in the reaction rates.

Chemistry of the DNA-Anthracycline Cross-Linking Reaction. A high-resolution structure of daunorubicin cross-linked to a guanine within a hexanucleotide has been presented.[3] Figure 9A shows the chemical structure of the cross-link between N2 of guanine and the 3′-NH_2 of doxorubicin.

Figure 9B sketches a plausible reaction mechanism for the formation of the cross-link. The well studied reaction of nucleophilic nitrogen with a carbonyl group is strongly influenced by a number of factors. It is generally accepted that the initial attack of basic nitrogen leads to the intermediate **I**, which can undergo intramolecular proton transfer to carbinolamine

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(hemiaminal) **II**. This is followed by a dehydration step giving iminium ion **IV** and then by loss of a proton to a neutral imine **V**. The electrophilic carbon of the imine can then react with the nucleophilic amino group of the second molecule, leading in consequence to the formation of an aminal structure. In studied examples, this could lead to the formation of a methylene bridge between the 3′-amino group of the anthracycline and the N2-amino group of the guanine (Figure 9A).

Evidence that the proximity of the 3′-NH_2 and N2 of guanine is one driving force behind the cross-linking reaction comes from the results obtained using **WP608**. Whereas the amino group in **WP608**, which is located at the C-4′ position, can react with HCOOH is a sequence of reactions **I**−**V** to imine **V**, intercalation places the C-4′-NH_2 too far away from guanine N2 to participate in aminal formation. As a result, no cross-linked product for **WP608** was observed.

Chemically Cross-Linked Daunorubicin Remains Intercalated. The high-resolution structure of HCOH-cross-linked daunorubicin obtained from X-ray crystallographic studies showed that the anthraquinone ring system was intercalated into DNA in the same way as the noncovalently bound drug.[3b] The results of our spectroscopic studies shown in Figures 4−6 indicate that, in solution, HCOH-cross-linked daunorubicin remains intercalated. The visible absorbance, fluorescence emission, and CD spectra are essentially identical for cross-linked and noncovalently bound daunorubicin. Intercalation of daunorubicin results in a pronounced induced circular dichroism band near 300 nm, a wavelength region for which absorbance is due to a transition dipole moment across the short axis of the anthraquinone ring system. The magnitude of this induced CD band is identical for both cross-linked and noncovalently bound daunorubicin, consistent with both forms being intercalated.

Both cross-linked and noncovalently bound daunorubicin increase the melting temperature of herring sperm DNA, although there are differences in the melting profiles. Noncovalently bound daunorubicin increases the T_m by 16 °C (Figure 6), and under the conditions of the melting experiment, the melting profile is distinctly biphasic. Such behavior is predicted by the theories of Crothers[9] and McGhee[10] and results from a redistribution of ligand over the course of the melting transition when drug is present at less than saturating concentrations. Interestingly, HCOH-cross-linked daunorubicin, at the same binding ratio, elevates the T_m to an even greater extent and lessens the biphasic character in the melting profile. The explanation for this is that the covalent attachment of the drug prevents its redistribution over the course of the DNA melting transition. The fact that the DNA duplex does melt with the cross-linked drug suggests, however, that the two DNA strands have not been cross-linked by HCOH. A limited number of experiments have been done to show that the cross-linked adduct is thermolabile. This complicates the detailed interpretation of melting experiments, since cross-links are probably broken, and drug released, at the higher temperatures reached near the end of the melting experiment. Nonetheless, melting experiments do offer an important qualitative characterization of adduct formation.

Comparison with Other Types of Anthracycline−DNA Cross-Linking Reaction. The covalent anthracycline−DNA reaction described here is distinct from the recently described interstrand Adriamycin−DNA cross-linking reaction.[11] That reaction appears to involve the drug chromophore and the N2 of guanine, whereas the cross-linking reaction described here clearly involves the 3′-NH_2 substituent. Further, the rate of HCOH cross-linking of daunorubicin to DNA is dramatically faster than the formation of interstrand cross-links between Adriamycin and DNA. The latter reaction requires 1−2 days, while the former reaction is complete within 20 min (Figure 1). Both the cross-linked adducts reported in ref 11 and those described here are heat labile.

Table 2. Chemical Cross-Linking of Daunorubicin to Deoxypolynucleotides of Defined Sequence

Polynucleotide	[DNA] μM bp	C_b^o μM	C_b^x μM
$[Poly(dGdC)]_2$	320.0	30.7	30.2
			30.6
Poly dG:Poly dC	275.0	25.0	24.5
			24.8
Poly dG:Poly dC	241.0	14.2	13.6
			14.2
Poly dA-dC:Poly dT-dG	182.0	13.8	13.1
			13.8
$[Poly(dIdC)]_2$	316.0	29.6	0.01
			0.02
$[Poly(dAdT)]_2$	357.0	36.1	0.00
			0.02

[a] Cross-linking reactions were conducted at 24 °C in solutions containing the indicated polynucleotide concentrations (in μM bp), sodium borate buffer, pH 8.2, and 2% (v/v) HCOH. After 30 min, reactions were stopped by phenol extraction or by the addition of SDS to a final concentration of 1.33% (italicized entry). C_b^o is the concentration of bound antibiotic at the start of the reaction. C_b^x is the concentration of antibiotic remaining bound (and presumably covalently cross-linked) to the DNA after SDS or phenol extraction.

The Requirement for N2 of Guanine. Studies of the cross-linking of daunorubicin to synthetic deoxypolynucleotides of defined sequence (Table 2) show that there is an absolute requirement for guanine. Since the daunosamine lies in the minor groove, we infer that guanine N2 is the key substituent, since it is accessible in the minor groove. The cross-linking reaction is therefore base specific, with an absolute requirement for guanine.

The results of Table 1 show that daunorubicin, doxorubicin, and WP711 are all quantitatively cross-linked to herring sperm DNA under the conditions used in those experiments. All of the initially bound drug becomes covalently cross-linked to the DNA. Further comment on this finding is in order in light of the guanine specificity of the cross-linking reaction. In the experiments reported in Table 1, DNA was added in large excess, with 0.1 mol drug/mol bp, i.e., one drug molecule for every ten base pairs. Herring sperm DNA has a G+C content of 42.2%, which means that 21.1% of the bases present in the DNA are guanine. Under the conditions of the experiments described in Table 1, we infer from the data that approximately half of the available guanine residues (∼0.1/0.211) are cross-linked to drug. Noncovalently bound daunorubicin normally preferentially binds to the triplet sequences 5′(A/T)GC or 5′-(A/T)CG.[2] The sequence preference of the formaldehyde cross-linked drug must, we assume, be different from this triplet and contain a guanine at the 5′ position instead of the A or T. The results of Table 1 show that all of the initially noncovalently bound drug is cross-linked, but for this to occur, we assume that drug molecules must redistribute from their otherwise preferred triplet site to ones containing guanine at the 5′ position.

Studies of the inhibition of restriction enzymes by cross-linked daunorubicin confirm the base specificity. Restriction enzymes

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probe cleavage at specific DNA sequences and may be used to characterize, at higher resolution, the specificity of the cross-linking reaction. The enzyme Nae I, whose recognition site is 5′GCCGGC3′, is completely inhibited by the cross-linking of daunorubicin. Since the recognition site contains all GC base pairs, this finding confirms the base specificity inferred from studies using deoxypolynucleotides. The enzyme Dra I, whose recognition site is 5′TTTAAA3′, is not inhibited at all at low binding ratios of cross-linked daunorubicin, and only one cleavage site is partially inhibited at higher cross-linking ratios. The weak inhibition observed is attributed to cross-linking of drugs to flanking sequences near that recognition site. The general conclusion from studies using restriction enzymes is that, within random DNA sequences, cross-linking remains guanine specific, and that drug will be selectively cross-linked to guanine sites within longer sequences.

The Cross-Linking Reaction is Regiospecific. Studies of the cross-linking of a series of anthracycline derivatives establish a clear regiospecificity for the reaction (Table 1). Both daunorubicin and doxorubicin are readily cross-linked by HCOH. Conversely, the aglycone, adriamycinone, can not be cross-linked, indicating that the needed reactive substituents are not present. Hydroxyrubicin, which lacks the 3′-NH_2, cannot be cross-linked, indicating the participation of that substituent in the reaction. Studies with WP608 and WP711 (Figure 7) further probe the regio- and stereoselectivity of the cross-linking reaction. In WP608, the NH_2 substituent is moved to the 4′ position, resulting in no cross-linking (Table 1). WP711 is a 3′-epimer of doxorubicin, having an amine at the 3′ position with altered stereochemistry. WP711 is cross-linked nearly as well as doxorubicin. Collectively, these results show that the NH_2 substituent at the 3′ position is absolutely required for efficient cross-linking. Moving the NH_2 group to the 4′ position results in loss of cross-linking reactivity. The cross-linking reaction is therefore regiospecific but not stereospecific.

The basis for the regiospecificity is clear from consideration of the high resolution crystal structures obtained for HCOH-cross-linked daunorubicin. Binding of the drug to DNA brings the 3′-NH_2 into close proximity to the N2 of guanine (within 4−6 Å), facilitating the cross-linking reaction. However, the NH_2 at the 4′ position is expected to protrude out of the minor groove and point away from its potential cross-linking partner on guanine. The efficiency of the cross-linking reaction is therefore due to the favorable proximity of the amine groups on the drug and DNA resulting from drug-DNA complex formation.

Implications for Drug Design. These studies suggest a new avenue for the rational design of novel anthracycline antibiotics that might form covalent adducts at specific sites within DNA. Incorporation of a reactive substituent at the 3′ position of the daunosamine that might react with N2 of guanine is a design strategy suggested by these studies. The design and synthesis of compounds according to this strategy is underway in our laboratory.

Summary

The results presented here establish that daunorubicin and doxorubicin can be rapidly and efficiently cross-linked to natural DNA in solution by HCOH. The reaction is base specific, with an absolute requirement for a guanine, and is regiospecific, with a requirement for an amine at the 3′ position of the daunosamine moiety. The cross-linked drug remains intercalated. These fundamental studies suggest new avenues for the rational design of new anthracycline antibiotics capable of forming covalent adducts with DNA.

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