Characterization of covalent Adriamycin-DNA adducts

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ABSTRACT Adriamycin is a popular antineoplastic agent whose ability to form covalent adducts with DNA has been correlated to cellular apoptosis (programmed cell death) in tumor models. We have isolated and purified this adduct formed under oxidoreductive (Fenton) conditions in Tris buffer. We show by homo- and heteronuclear NMR spectroscopy that the covalent Adriamycin-DNA adduct is structurally equivalent to that resulting from direct reaction with formaldehyde. Covalent linkage of the drug to one of the DNA strands confers remarkable stability to the duplex, indicated by a 162-fold reduction in the rate of strand displacement compared with the complex with noncovalently bound drug. Glyceraldehyde also engenders covalent Adriamycin-DNA complexes, providing a possible relevant biological context for in vivo adduct formation.

Of the approximately 50 drugs used in chemotherapy regimens, doxorubicin (tradename, Adriamycin) has found one of the widest applications because of its activity against a broad range of malignancies (1). Despite a large body of evidence that Adriamycin acts dominantly at the DNA level through supercoiling (59:1 acrylamide/N,N'-methylenebisacrylamide) polyacrylamide containing 80 mM Tris Borate (pH 7.5) and 1 mM EDTA. Gel type A was 20% (59:1 acrylamide/N,N'-methylenebisacrylamide) polyacrylamide containing 8 mM urea, 80 mM Tris borate (pH 7.5), and 1 mM EDTA.

DNA Purification. After 1-μmol-scale syntheses, the crude DNA reaction products were purified on gel type B (20 × 40 cm) at 1.25 W/cm by electrophoresis. The desired band was visualized by UV shadowing, excised from the gel, and eluted into 100 ml of water overnight at ambient temperature. This solution was filtered to remove particulate acrylamide before loading onto a PerSeptive Biosystems HQ-20 anion-exchange HPLC column. After elution with 1 M NaCl, the appropriately 4-ml samples were dialyzed overnight against two changes of 4 liters of double-distilled H2O and were subse-

This paper was submitted directly (Track II) to the Proceedings office. Abbreviations: NOESY, nuclear Overhauser effect spectroscopy; HSCC, heteronuclear single quantum correlation; vdl, virtual cross-link.

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quently dried in a Speed-Vac. Purified DNA pellets were resuspended in 0.2–0.5 ml of buffer A.

**Spectrophotometric Quantitation of Adriamycin and DNA.**

A molar extinction coefficient of 11,500 M$^{-1}$cm$^{-1}$ at 480 nm was used for free Adriamycin; for covalently bound Adriamycin, the molar extinction coefficient used was 7,677 M$^{-1}$cm$^{-1}$ at 506 nm. The molar extinction coefficient used for the self-complementary hexamer d(ATGCAT)$_2$ in the NMR experiments was 120,800 M(dup)$^{-1}$cm$^{-1}$ at 260 nm. For the non-self-complementary DNA in the strand-exchange reactions, the following extinction coefficients were used:

- $5^\prime$-ATTATTGTTATTA-3$'$, 138,300 M$^{-1}$cm$^{-1}$ for 5$'$-ATTTATGCTTATTA-3$'$.
- $5^\prime$-TAATAAGCATAAAT-3$'$, 152,500 M$^{-1}$cm$^{-1}$.

**Formation of Covalent Adriamycin-DNA Adducts.**

**Formation with CH$_2$O.** Multiple 15-ml reactions were run in parallel. Each contained 4.2 mM d(ATGCAT)$_2$, 10 mM Adriamycin, 75 mM FeCl$_3$, 7 mM DTT, and 40 mM Tris HCl (pH 7.0). Reactions were run at 10°C for 1 week, the time determined for nearly 100% conversion of all DNA to covalent species.

**Formation with $^{13}$CH$_2$O.** $^{13}$C-labeled Adriamycin-DNA adducts were formed as described above for CH$_2$O except that $^{13}$CH$_2$O was used in place of CH$_2$O.

**Formation with CD$_2$O.** Adriamycin-DNA adducts containing a deuterated methylene bridge were formed as described above for CH$_2$O except that CD$_2$O was used in place of CH$_2$O, and the reactions were carried out in 99% 2H$_2$O instead of in H$_2$O.

**Formation with Glyceraldehyde.** The glyceraldehyde-mediated adduct was formed under identical conditions, except that CH$_2$O was replaced by glyceraldehyde, and only one reaction was run for analytical purposes.

**NMR Sample Preparation.** After the reaction, volumes were pooled into centrifugal concentrators, concentrated to approximately 0.2 ml of buffer A. Electrophoretic separation of Adriamycin-DNA adduct away from unreacted DNA followed overnight at 4°C and 2 W, after which the adduct was clearly visible to the naked eye as a pink band on the gel. Rapid electroelution of the adduct into buffer C at 4°C using barriers fashioned from dialysis membrane (molecular weight cutoff, 1,000) yielded pure adduct. This was again centrifugally concentrated at 4°C to approximately 0.2 ml, dialyzed into buffer A at 4°C, lyophilized, and resuspended in 0.2 ml 100% 2H$_2$O.

**NMR Spectroscopy.** One- and two-dimensional NMR experiments were performed on a Varian Unity model 500 MHz spectrometer at 10°C on a triple resonance (H, C, N) probe. A standard two-dimensional nuclear Overhauser effect spectroscopy (NOESY) pulse sequence provided by Varian was used for all homonuclear experiments. A sweep width of 4,000 Hz with 1,024 complex data points was used for all $^1$H spectra. For each of 300 $t_1$ increments, 16 transients were acquired, each with a recycle delay of 7 s. The mixing time was 0.25 s. After acquisition, data were transferred to a Silicon Graphics INDY.

**FIG. 2.** The 500-MHz $^1$H and HSQC NMR spectra of covalent Adriamycin-DNA adducts at 10°C. (a) Comparison of CH$_2$O- and Tris/Fe(III)/DTT-mediated Adriamycin-DNA adducts by $^1$H NMR indicates that the two are structurally equivalent. (b) Portion of the carbon HSQC spectrum of $^{13}$CH$_2$O-mediated Adriamycin-DNA. The methylene $^{13}$C has strong cross-peaks to each of its nonequivalent geminal protons, which in turn bear cross-peak arrays structurally consistent with the incorporation of CH$_2$O into the Adriamycin-DNA adduct as shown in Fig. 1.
mediated Adriamycin-DNA adducts were formed on the self-
that adduct formation under Fenton conditions with Tris
on a Fuji PhosphorImager, data were analyzed by using
After gel resolution, gels were covered with Saran Wrap and
containing purified adduct) at 10°C.
tions with DNA alone) or on gel type B (for incubations
with noncovalent Adriamycin-DNA complexes and incuba-
times and frozen until resolution on gel type A (for incubations
strand. Aliquots were removed from the incubations at various
times and frozen until resolution on gel type A (for incubations
containing purified adduct) at 10°C.
Quantitative and Analysis of Strand-Exchange Products.
After gel resolution, gels were covered with Saran Wrap and
allowed to expose a Fuji BasIII imaging plate. After imaging
on a Fuji PhosphorImager, data were analyzed by using
MACBAS software (Version 1.01). Values for the fractional
amount of exchange at time t were calculated as the baseline
corrected ratios of quantitated double- to single-stranded
band intensities (I_d/I_s) on the gels. Strand-exchange rates were
then calculated as d(I_d/I_s)/dt. For the reversibly bound
duplex at 30 μM, strand-exchange rates at 0, 10, 20 and 40 μM
Adriamycin were calculated and replotted as d(I_d/I_s)/dt vs.
Adriamycin/duplex, allowing interpolation to the strand-
exchange rate at one Adriamycin/duplex (stoichiometrically
comparable to the covalent adduct). This then served as a
comparison to free and covalently bound DNA species.

RESULTS AND DISCUSSION
Comparison of Tris/Fe(III)/DTT- and CH2O-Mediated
NMR Spectra. Our ability to produce and purify large amounts
of stable covalent Adriamycin-DNA adduct in solution has
allowed a more direct structural comparison of these adducts
than previously possible by MS. CH2O-and Tris/Fe(III)/DTT-
mediated Adriamycin-DNA adducts were formed on the self-
complementary hexanucleotide d(ATGCAT)_{12}, containing
only a single potential site for adduct formation. Isolation
and purification of the respective adduct and analysis by high-field
1H NMR indicated that the CH2O- and Tris/Fe(III)/DTT-
mediated Adriamycin-DNA adducts are structurally identical
(Fig. 2a). These direct structural data show unambiguously
that adduct formation under Fenton conditions with Tris
buffer proceeds via CH2O, itself a product of the radical
disproportionation of Tris (14). Furthermore, the retention of
Adriamycin carbohydrate resonances in the adduct spectrum
displays the notion that Adriamycin-DNA adduct formation
under reductive conditions must proceed via formation of an
electrophilic quinone methide intermediate at Adriamycin C7
with concomitant reductive elimination of the daunosamine
sugar (15).

Fig. 3. Anomeric-aromatic portion of the 500-MHz 1H NOESY
spectrum at 10°C of the Adriamycin-DNA adduct mediated by Tris/
Fe(III)/DTT. The connectivity patterns between anomic and aro-
matic DNA protons are correlated by color to the schematic Adria-
mycin-DNA adduct shown above. The c-strand is shown in blue,
whereas the n-strand is depicted in black and red. The NOESY
connectivity pattern between DNA protons is interrupted between
the C1pA5 step of the c-strand and the T8pG9 step of the n-strand,
indicating intercalation of the Adriamycin chromophore at this point
and covalent attachment to G3. Connectivity along the c-strand is
completed by contacts between the aromatic protons C4H6 and ASH8
to the Adriamycin-H1' carbonyl group. The box representing
connectivity between the protons G9H8, G9H1', C10H6, and C10H5,
shown in green, was a characteristic feature of all observed Adriam-
ycin-DNA spectra of the type d[(AT)_{12}GC(AD)_{12}]. Residual unlabeled
cross-peaks represent small amounts of untreated DNA that was
excised from the gel with the adduct.

Incorporation of 13C-labeled CH2O. This finding corrobo-
rates earlier MS data with direct structural evidence that
CH2O is the essential species required for covalent Adriamy-
clin-DNA adduct formation. To demonstrate that CH2O is
itself incorporated into the covalent complex and to gain
insight as to the locus of this incorporation, the covalent adduct
was formed and isolated as described above with isotopically
labeled 13CH2O. If the aminal linkage between Adriamycin
and DNA were in fact to derive from CH2O, as suggested by
x-ray studies, then the adduct resulting from incorporation of
13CH2O would be expected to bear the 13C at the methylene
center of this linkage. The carbon HSQC spectrum of the
13CH2O-mediated Adriamycin-DNA adduct is shown in Fig. 2b
with its strong J-coupled cross-peaks between the methylene
13C and each of its (nonequivalent) geminal protons at 4.08
and 5.13 ppm. In independent two-dimensional 1H NOESY spec-
tra, these protons show NOE cross–peaks to the following
assigned drug and DNA protons: 5'-MeAdriamycin (1.35 ppm),
C10H2’dNA (1.97 ppm), A11H2’dNA (8.34 ppm), A11H8’dNA
(8.39 ppm), and C10H6’dNA (7.66 ppm). Atom labels are as
shown in Fig. 3 for the DNA and in Fig. 1 for Adriamycin.
These through-space contacts are consistent with the x-ray
model, indicating unambiguously that the aminal linkage
joining Adriamycin and DNA indeed derives from CH2O.
The identity of these methylene protons was further corroborated

Workstation and processed with the software package FELIX95
(Biosym Technologies, San Diego, CA). In general, processing
to the deconvolution of the H2HO signal, apodization with a
dine-four squared function and zero filling to 1,024 data points
before Fourier transformation. The heteronuclear 1H–13C
model, indicating unambiguously that the aminal linkage
joining Adriamycin and DNA indeed derives from CH2O.
The identity of these methylene protons was further corroborated

by the abolition of the above cross-peaks in the two-dimensional $^1$H NOESY spectrum of the adduct formed with C$_2$H$_2$O. The mutual cross–peak between the two methylene protons was similarly abolished in the spectrum of the C$_2$H$_2$O-mediated adduct.

**Spectroscopic Characteristics of the Adduct.** Fig. 3 shows the anomic-aromatic portion of the two-dimensional $^1$H NOESY spectrum of the Adriamycin-DNA adduct formed on d(ATAAGCATAAT)$_2$. From the connectivity pattern in this region, it is clear that only one reaction product is present, in which a single Adriamycin molecule is covalently bound per DNA duplex. Because the DNA oligomer is self-complementary before adduct formation, covalent Adriamycin binding to either strand generates an identical product. In this way, once either strand has become the c-strand through covalent linkage to Adriamycin (and by default the opposite strand has become the n-strand with noncovalent contacts to the drug), the dyad symmetry of the molecule is lost, and the absence of additional GpC sites precludes further adduct formation. The location of the intercalated Adriamycin chromophore and NOESY cross–peaks between Adriamycin and DNA protons is consistent with x-ray analysis, implying that the Adriamycin-DNA adduct in solution is structurally comparable to the crystal species, with Adriamycin covalently bound only to the c-strand and that the immense stabilization of the duplex against denaturation derives from the specific noncovalent interactions between Adriamycin and the opposite n-strand. Because of its extreme stability to denaturation, the term “virtual cross–link” (vxl) has been proposed to describe this monocovalent species (9, 10).

The interruption of anomic-aromatic cross-peak connectivity between bases C$_4$A$_5$ and bases T$_8$G$_9$ indicates that the chromophore of Adriamycin is intercalated 3' adjacent to the central GpC step on the c-strand and 5' adjacent to this domain on the n-strand. To date, x-ray and MS studies have used self-complementary oligonucleotides of the type d[(GC)$_2$], and d[(CG)$_2$], because it was assumed that intercalation of the drug chromophore between guanine and cytosine was a prerequisite for Adriamycin-DNA adduct formation. Fig. 3 shows that Adriamycin need not intercalate between GpC or CpG to form a stable covalent adduct with DNA. Rather, Adriamycin intercalation, which occurs adjacent to such domains, seems to be the primary determinant in fostering the covalent and noncovalent interactions necessary for duplex stabilization by the drug.

**Stability of Adduct via Strand-Exchange Kinetics.** With direct solution-based structural evidence establishing the importance of CH$_2$O in the formation of the vxl, irrespective of reaction conditions, we sought to quantitatively evaluate the high duplex stabilization caused by noncovalent contacts to the n-strand. As with the NMR studies, it was also important in this experiment to be able to rely on the stability of the purified adduct in solution over time. Duplex stabilization energy was measured as a function of labeled n-strand incorporation by exchange into (i) the duplex alone, (ii) the duplex intercalated (but not covalently reacted) with Adriamycin, and (iii) the purified vxl duplex. If the Adriamycin-DNA adduct were truly monocovalent to only the c-strand, one would expect some measurable exchange of an additional n-strand into the adduct without loss of vxl character. The rate of this exchange as compared with unintercalated and intercalated duplexes of identical sequence would yield a measure of the kinetic duplex stabilization afforded by, respectively, the entire vxl complex and the noncovalent contacts to the n-strand alone.

Strand-exchange rate, and, therefore, the duplex stabilization energy, was found to vary markedly with DNA species. Experimental results for strand-exchange incubations of duplex DNA with and without a single vxl lesion are shown in Fig. 4, and the respective strand-exchange rates are summarized in Table 1.

The time-dependent incorporation of labeled n-strand into the vxl duplex without loss of vxl character is itself cogent evidence that the Adriamycin in this adduct is covalently bound to only one DNA strand rather than to each strand, as would be expected for a classical DNA cross-link. One molecule of intercalated but unreacted Adriamycin stabilizes the duplex by 3.9-fold with respect to unintercalated DNA (Fig. 4). The corresponding stabilization by one covalent Adriamycin-DNA adduct over unintercalated DNA is 637-fold. Alternatively, specific noncovalent interactions between Adriamycin and the n-strand in the vxl species result in a duplex stabilization 40-fold greater than that afforded by simple intercalation.

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**Table 1. Summary of strand exchange data from Fig. 4 for various species**

<table>
<thead>
<tr>
<th>DNA species</th>
<th>$k$, hr$^{-1}$</th>
<th>Relative $k$</th>
</tr>
</thead>
<tbody>
<tr>
<td>vxl adduct</td>
<td>$1.25 \times 10^{-4}$</td>
<td>1</td>
</tr>
<tr>
<td>One intercalated Adr/duplex</td>
<td>$2.02 \times 10^{-2}$</td>
<td>161.6</td>
</tr>
<tr>
<td>Unintercalated DNA</td>
<td>$7.97 \times 10^{-2}$</td>
<td>637.6</td>
</tr>
</tbody>
</table>

*Interpolated value determined in an independent series of strand-exchange incubations containing various concentrations of reversibly bound Adriamycin.*

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Fig. 4. Results of kinetic strand-exchange experiments with unintercalated DNA (○), purified Adriamycin-DNA adduct (□), and DNA intercalated with 1 Adriamycin/duplex. The latter is shown as a dashed line and represents the rate estimated by interpolation from a separate series of strand-exchange experiments containing various concentrations of noncovalently bound Adriamycin. Strand-exchange rate constants are summarized in Table 1. The DNA used in the kinetic strand-exchange experiments was the 14-bp oligomer d(TAATAGCATAAT)-d(ATTATGCTATTA), which contains a single potential site for adduct formation.

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Fig. 5. Mediation of stable Adriamycin-DNA adduct formation by glyceraldehyde. Reactions were in buffer A (13), and resolution was on gel type B (13). Reactions contained 100 μM Adriamycin and 100 μM oligomer d(uppercase:AAAGCTTTT), and were run overnight at 25°C. Lanes: 1, only DNA and Adriamycin; 2, DNA, Adriamycin, and 0.4% CH$_2$O; 3, DNA, Adriamycin, and 0.5% glyceraldehyde. The gel was photographed by UV shadowing. Although not as efficient an initiator of stable Adriamycin-DNA adducts as CH$_2$O, glyceraldehyde mediates stable adduct formation in high enough yield to be of potential biological significance.
tion. Presumably, noncovalent interactions between Adriamycin and the n-strand are facilitated by immobilization of the drug attending its covalent attachment to the c-strand.

Independent experiments comparing the denaturation propensity for purified vxl adducts and unreacted unintercalated duplexes of various sizes have estimated the total vxl stabilization over Adriamycin-free DNA to be worth between six A-T base pairs and four G-C base pairs (data not shown). Total vxl duplex stabilization by this measure is, therefore, energetically equivalent to approximately 12 interstrand DNA H-bonds.

**Adriamycin-DNA Adducts Mediated by Glyceroldehyde.** It is very likely that this surprising degree of vxl duplex stabilization against denaturation is related to the ability of covalent Adriamycin-DNA complexes to persist in vivo. Various mechanisms for the in vivo production of CH2O have been proposed, including autocatalytic Bayer–Villiger oxidation at Adriamycin C13 by H2O2 (9, 10), and oxidation of DNA-associated polyamines such as spermine by hydroxide radical (9, 10). Each scenario is dependent on the ability of Adriamycin to bind Fe(III) with extremely high affinity (16, 17) and on the availability of cellular reduction potential in the form of glutathione or xanthine oxidase, which is required for iron redox cycling leading to reactive oxygen species. In addition, we have found that glyceroldehyde is sufficient to engender Adriamycin-DNA complexes stable to denaturation (Fig. 5). In this case, the reaction proceeded only at ambient temperatures. Although the more biologically accessible 3-phosphate derivative of glyceroldehyde (G3P) failed to elicit stable Adriamycin-DNA adducts, it is unknown whether this inability is genuine or, rather, due to the instability of G3P at the ambient temperatures required for reaction. Similar attempts using acetaldehyde, erythrose, pyridoxal, and pyridoxal 5-phosphate as potential vxl mediators did not yield significant levels of vxl species.

The ability to isolate stable covalent Adriamycin-DNA adducts opens up new experimental routes for the structural evaluation and comparison of covalent complexes formed under the biologically relevant conditions proposed above, as well as under conditions not yet recognized. Of greatest initial importance will be the question of whether CH2O is a prerequisite for adduct formation in vivo or whether comparable duplex stabilization might also be effected by some other, hitherto unsuspected, cellular species or process.

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