Redox Pathway Leading to the Alkylation of DNA by the Anthracycline, Antitumor Drugs Adriamycin and Daunomycin

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Reaction of the anthracycline, antitumor drugs adriamycin and daunomycin with the self-complementary DNA oligonucleotide GCGCGCGC, $(GC)_4$, in the presence of the reducing agent dithiothreitol, the oxidizing agent hydrogen peroxide, or the alkylating agent formaldehyde gives a similar mixture of DNA−drug adducts. Negative ion electrospray mass spectra indicate that adduct formation involves coupling of the DNA to the anthracycline *via* a methylene group and that the major adduct is duplex DNA containing two molecules of anthracycline, each bound to a separate strand of the DNA *via* a methylene group. The source of the methylene group is formaldehyde. A molecular structure with each anthracycline intercalated at a 5′-CpG-3′ site and covalently bound from its 3′-amino group to a 2-amino group of a 2′-deoxyguanosine nucleotide is proposed based upon spectral data and a relevant crystal structure. The reaction of $(GC)_4$ with the anthracyclines and formaldehyde forms an equilibrium mixture with DNA−drug adducts which is shifted toward free DNA by dilution. The results suggest a pathway to the inhibition of transcription by reductively activated adriamycin and daunomycin. Reductive activation in the presence of oxygen yields hydrogen peroxide; hydrogen peroxide oxidizes constituents in the reaction mixture to formaldehyde; and formaldehyde couples the drug to DNA. In this regard, hydrogen peroxide reacts with adriamycin *via* Baeyer−Villiger reactions at the 13-position to yield **2**, **3**, and formaldehyde. Formaldehyde also results from hydrogen peroxide oxidation of Tris [tris(hydroxymethyl)aminomethane] present in transcription buffer and spermine, a polyamine commonly associated with DNA *in vivo*, presumably *via* the Fenton reaction.

Introduction

Adriamycin (doxorubicin) continues to be one of the most important antitumor drugs in the clinic. It is a broad spectrum drug particularly useful in the treatment of Hodgkin's disease, non-Hodgkin lymphomas, acute leukemias, sarcomas, and solid tumors of the breast, lung, and ovary.[1] Daunomycin (daunorubicin), another anthracycline antitumor drug which differs from adriamycin only by the absence of a hydroxyl group at the 14-position, has been used primarily for the treatment of leukemia; however, a new liposomal formulation, DaunoXome, may extend its application.[2]

Both drugs have been investigated extensively with respect to mechanism of action. The drugs are strong intercalators in DNA with the A-ring and amino sugar moieties having important interactions with the minor groove. This activity results in topoisomerase-induced DNA strand breaks.[3,4] The quinone functionality is redox active and catalyzes the production of reactive oxygen species in the presence of a reducing agent and molecular oxygen.[5,6] Production of reactive oxygen species has been linked to both tumor cell cytotoxicity and cardiotoxicity. In the absence of molecular oxygen, reduction leads to glycosidic cleavage with the production of a quinone methide transient (Scheme 1) which shows mild electrophilic and nucleophilic reactivity.[7] Alkylation of DNA has been proposed as a possible cytotoxic event; however, no structural evidence for its occurrence has been reported. The quinone methide from reductive activation of the related anthracycline, menogaril, has been shown to react slowly with 2′-deoxyguanosine at its 2-amino substituent in a strictly anaerobic medium.[8] The predominant reaction of the quinone methide from reduction of adriamycin and daunomycin is with the proton, leading to the respective, inactive 7-deoxyaglycons.

Recent experiments by Phillips and co-workers have refocused attention on the possibility of reductive activation leading to the alkylation of DNA as an important cytotoxic event. They noticed that adriamycin in the presence of ferric ion and dithiothreitol caused transcriptional blockages in DNA.[9,10] Adriamycin is a strong chelator of ferric ion, and complexation was proposed as necessary to achieve reduction with DTT. Transcriptional blockages were specific for 5′-GpC-3′ sites in the DNA and appeared to be the result of covalent bond formation at the 2-amino group of the deoxyguanosines.[9,11] The covalent bonds were labile upon isolation of the DNA followed by redissolution in buffer.[12] They proposed the quinone methide as a possible reactive intermediate based upon the precedent of reaction of reductively activated menogaril with 2′-deoxyguanosine. An inconsistency with the quinone methide as a reactive intermediate was an insensitivity to the presence of molecular oxygen.[9]

In a communication,[13] we reported that the covalent bonds between adriamycin or daunomycin and DNA at 5′-GpC-3′ sites were the result of formaldehyde Schiff base chemistry linking the 3′-amino group of the anthracyclines to the 2-amino substituent of deoxyguanosines. Further, the formaldehyde could result from anthracycline redox chemistry. We now report more

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Scheme 1

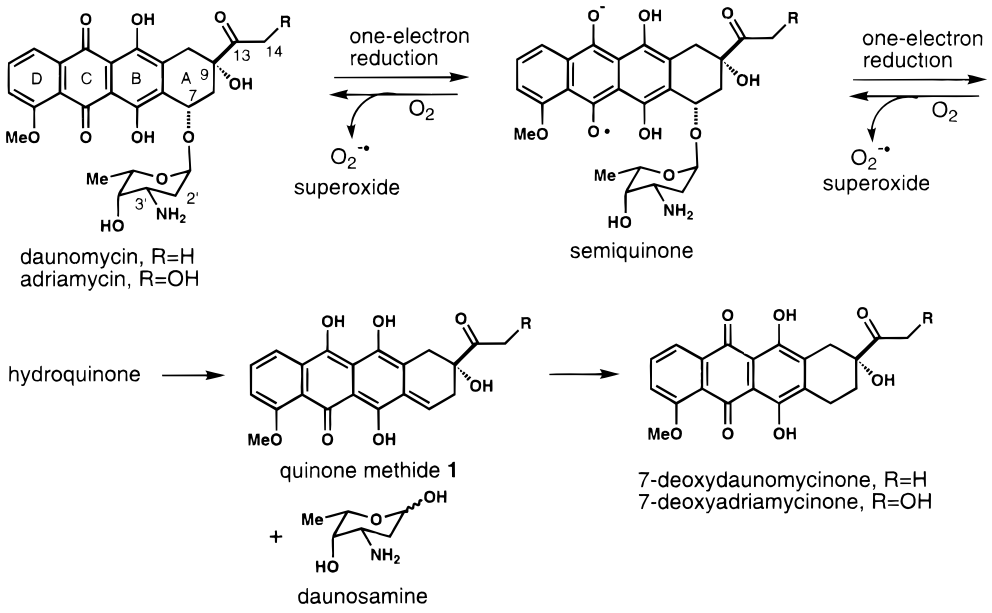


Table 1. Formation of DNA−Drug Adducts at Ambient Temperature as a Function of Reaction Conditions

entry	drug	buffer reagent (equiv)	reaction time	DNA ratios $(GC)_4$:1:(2 + 3):4:(5)[a]
1	adriamycin	Tris (pH 8.0)/KCl/EDTA/$MgCl_2$ $FeCl_3$/DTT (∼400 equiv)	4 days	0:19:35:45
2	adriamycin	Tris (pH 7.4)/KCl/EDTA/$MgCl_2$ H_2O_2 (∼30 equiv)	4 days	40:5:20:35
3	adriamycin	phosphate (pH 8.0)/$MgCl_2$ H_2O_2 (∼20 + 30 equiv)[b]	14 days	83:8:6:1:(2)
4	adriamycin	phosphate (pH 7.0)/$MgCl_2$ H_2O_2 (∼50 equiv)	12 days	60:11:16:5:(8)
5	adriamycin	Tris (pH 7.4)/KCl/EDTA/$MgCl_2$ formaldehyde (10 equiv)	1 day	0:8:30:62
6	daunomycin	Tris (pH 8.0)/KCl/EDTA/$MgCl_2$ H_2O_2 (∼12 equiv)	9 days	trace:12:36:48
7	daunomycin	Tris (pH 7.4)/KCl/EDTA/$MgCl_2$ formaldehyde (5 equiv)	1 h[c]	40:9:34:17

[a] DNA ratios were determined from integrals of chromatograms for detection at 260 nm. They are uncorrected for differences in response. [b] Hydrogen peroxide was added in two portions with the second portion added after 6 days. [c] Complete disappearance of the DNA was observed after 5 h with adducts 3 and 4 as the major products.

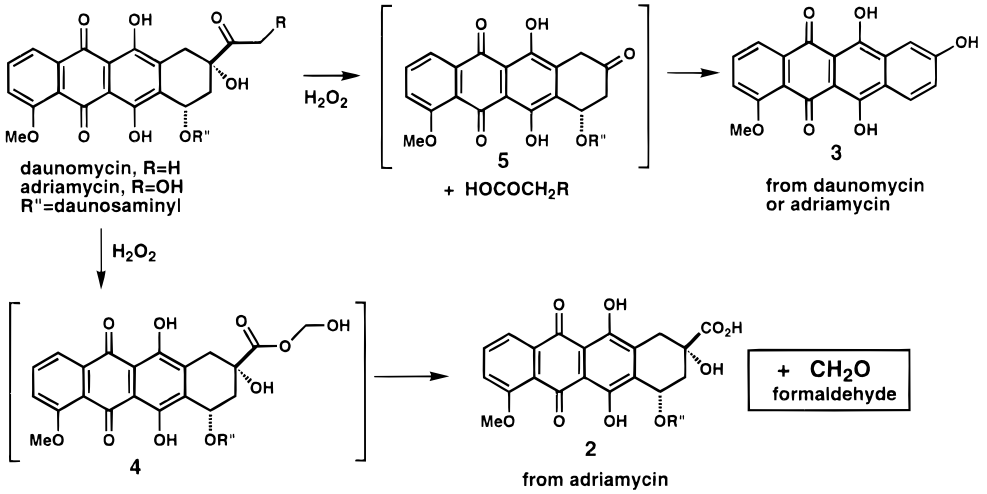
data and the full experimental details for the earlier experiments and the results of some additional relevant experiments, and we discuss the results more extensively with respect to other observations in the literature.

Results

Formation of DNA−Adriamycin and DNA−Daunomycin Adducts. The DNA selected for these studies was $(GC)_4$ because it is self-complementary, bears multiple 5′-GpC-3′ sites, and is small enough for mass spectral characterization of labile drug−DNA adducts. Further, $(GC)_4$ has a melting temperature sufficiently high to maintain a predominantly double-stranded structure in both the reaction medium and the eluent used for HPLC analyses and isolations. The predominant reaction medium was the transcription buffer employed by Phillips and co-workers, pH 8 Tris/KCl/EDTA/$MgCl_2$, and the HPLC eluent was 90% 20 mM triethylammonium acetate/10% acetonitrile. The respective $(GC)_4$ melting temperatures were 61 and 45 °C.

Reaction of $(GC)_4$ with adriamycin in pH 7.4 or 8.0 Tris buffer containing ferric chloride and dithiothreitol at 25 °C was complete in 4 days and gave a product mixture which showed four HPLC peaks designated 1−4, corresponding to DNA−drug adducts 1−4 (see Figure 4 in Supporting Information for an example of a chromatogram). Peaks for adducts 2 and 3 were usually poorly resolved and, consequently, integrated together. The ratio of DNA to adducts is reported for pH 8.0 in Table 1 (entry 1). Neither the extent of reaction as a function of time nor the adduct ratios showed significant dependence on the pH in the region 7−8. UV−vis spectra of the adducts showed a λ_{max} for the drug at 510 nm consistent with the chromophore intercalated in double-stranded DNA (dsDNA). The relative intensities of the absorptions at 260 and 480 nm indicated that adducts 1 and 2 contained one drug chromophore per dsDNA and adducts 3 and 4, two drug chromophores per dsDNA. Since adriamycin redox chemistry is very sensitive to the presence of molecular oxygen, we surmised that the earlier conclusion of Phillips and co-workers that molecular oxygen was not a factor in the reaction was incorrect.[9] A reaction similar to entry 1 of Table 1 but degassed under high vacuum, using freeze−thaw techniques, showed no reaction of the DNA after 4 days. With exposure to air, the DNA−drug adduct mixture appeared after a subsequent reaction period. The requirement of molecular oxygen indicated

Scheme 2



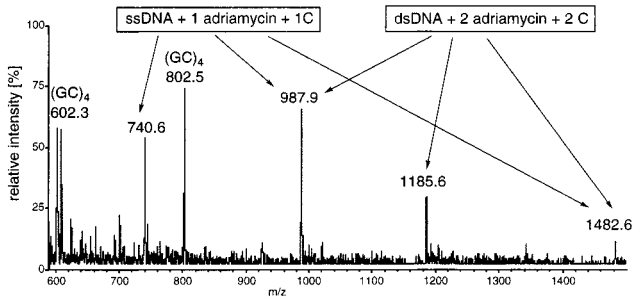


Figure 1. Negative ion electrospray mass spectrum (ESMS) of DNA−adriamycin adduct 4 from reaction of $(GC)_4$ with adriamycin and hydrogen peroxide in Tris buffer. Peaks representing assigned ions bearing one or more sodium or potassium ions are not labeled to simplify the figure. Calculated mass to charge ratios appear in Table 4 in Supporting Information.

that the catalytic production of reactive oxygen species by adriamycin was important. When the reaction was performed with hydrogen peroxide in place of $FeCl_3$/DTT, the same four HPLC peaks for DNA−drug adducts were observed, although the yield was lower after a comparable time period (see Table 1, entry 2). This was an early clue that the role of iron went beyond raising the reduction potential of adriamycin as originally proposed.[9] Replacement of Tris buffer with phosphate buffer made the reaction even slower and produced a fifth DNA−drug adduct, designated 5, as shown in Table 1, entries 3 and 4. Hence, Tris appeared to play a role beyond that of a buffer.

At this point mass spectral analysis of the predominant DNA−drug adduct (4) from reaction in Tris buffer with hydrogen peroxide was employed to characterize the molecular nature of the covalent bond. A major problem was the reported and observed instability of the DNA−drug adducts with respect to isolation. HPLC separation followed by centrifugal evaporation of the eluent, acetonitrile/pH 6 triethylammonium acetate, yielded only recovered $(GC)_4$ and adriamycin. Addition of pH 7 phosphate buffer prior to centrifugal evaporation stabilized the DNA−drug adducts. The negative ion electrospray mass spectrum (ESMS) is displayed in Figure 1. It shows multiple ions corresponding to double-stranded DNA bound to two molecules of adriamycin each by a methylene unit, single-stranded DNA bound to one molecule of adriamycin by a methylene, and DNA. The calculated masses corresponding to each peak are provided in Table 4 which appears in Supporting Information. We interpret the spectrum in terms of adduct 4 being double-stranded DNA covalently bound to two molecules of adriamycin, each by a methylene group. The other peaks result from decomposition in the inlet system of the mass spectrometer during ionization. The interpretation was further supported by MS/MS fragmentation experiments on *m/z* 740.6 which yielded ions for DNA, DNA bound to a single carbon, and adriamycin bound to a single carbon. Subsequently, mass spectra of DNA−drug adducts 1, 2 + 3, and 4 from reaction in Tris buffer with $FeCl_3$/DTT were obtained; they are shown in Figure 5 of Supporting Information. They all show peaks for DNA bound to adriamycin by a methylene group as well as a peak for adriamycin bound to a single carbon.

An important clue to the nature of the covalent bond linking adriamycin to DNA came from parallel studies of the reaction of adriamycin with DTT and with hydrogen peroxide. Both reactions gave two products, isolated and identified as the carboxylic acid **2** and the fully aromatized aglycon **3** (Scheme 2). The structure for the carboxylic acid was established from spectral comparison with a sample prepared by periodate oxidation[14] and for the aglycon **3**, from spectral comparison with literature data.[15] The reactions were characteristic of the Baeyer−Villiger oxidation at the 13-position *via* transients **4** and **5** as proposed in Scheme 2. The Baeyer−Villiger mechanism predicted that the byproduct of formation of **2** was formaldehyde. In fact, formaldehyde was detected in the reaction mixture using the Hantzsch reagent.[16] The corresponding Baeyer−Villiger oxidation of daunomycin with hydrogen peroxide yielded the aglycon **3** but not the carboxylic acid **2**.

Quite by accident, Wang and co-workers discovered that formaldehyde couples daunomycin to DNA *via* Schiff base chemistry involving the 3′-amino group of the drug and the 2-amino group of 2′-deoxyguanosines.[17] In fact, they obtained single crystals of a DNA−drug adduct using $(CG)_3$ for the DNA and performed an X-ray structural characterization. The adduct has two molecules of drug bound to double-stranded DNA, one to each strand *via* a one-carbon linkage (Scheme 3). This structure suggests a parallel structure for one of the DNA−drug adducts, probably adduct 4, from the reaction of $(GC)_4$ with adriamycin and hydrogen peroxide

Scheme 3

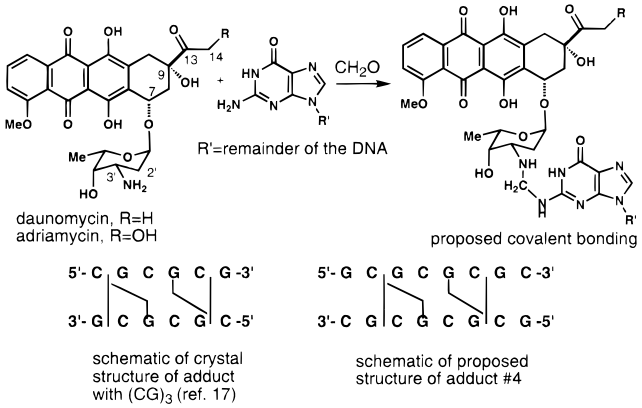


Table 2. Formation of Formaldehyde from Reaction of Tris Buffer (40 mM Tris/100 mM KCl/3 mM $MgCl_2$) with 20 mM Hydrogen Peroxide as a Function of Conditions and Time

	[formaldehyde] (μM) at time		
buffer	2 h	10 h	69 h
Tris (pH 7.4)	6.0	8.4	26
Tris (pH 8.0)/EDTA (0.10 mM)			1.0
Tris (pH 7.4)/Fe^{2+} (50 μM)	80	110	180
Tris (pH 8.0)/EDTA (0.10 mM)/ Fe^{2+} (50 μM)	1.5	38	1600
Tris (pH 7.4)/Fe^{3+} (50 μM)	9.1	6.0	84
Tris (pH 8.0)/EDTA (0.10 mM)/ Fe^{3+} (50 μM)	3.0	18	1500

as shown in Scheme 3. Other possible adduct structures bearing one and two adriamycins based upon the Wang model are proposed in Figure 9 of Supporting Information. Wang and co-workers subsequently solved the crystal structures of several additional DNA−anthracycline adducts formed with formaldehyde.[18,19]

Reaction of daunomycin with $(GC)_4$ and hydrogen peroxide in Tris buffer also yielded four DNA−drug adducts as shown in Table 1 (entry 6). A mass spectrum of adduct 4 appears in Figure 1 of the earlier communication[13] and shows peaks for double-stranded DNA bound to two molecules of daunomycin, each by a methylene unit. However, daunomycin does not yield formaldehyde upon Baeyer−Villiger oxidation. What is the source of the formaldehyde? The answer lies in the enhanced DNA−drug adduct formation in Tris buffer, especially with iron present (Table 1, entries 1−4). In fact, reaction of Tris with hydrogen peroxide in the presence of Fe(II) or Fe(III) produced a significant amount of formaldehyde and even a larger amount when EDTA was present, as shown in Table 2. This appears to be a Fenton reaction and is precedented by the Cu(II)/EDTA-catalyzed oxidation of Tris to formaldehyde.[20]

Reaction of $(GC)_4$ with adriamycin or daunomycin with excess formaldehyde rapidly gave the same four DNA−drug adducts as indicated by HPLC (Table 1, entries 5 and 7). Both reactions were complete in less than 1 day using 5−10 equiv of formaldehyde. Isolation and mass spectral analysis indicate similar structures as shown for daunomycin in Figure 6 of Supporting Information. One exception is the appearance of peaks corresponding to single-stranded DNA bound to two molecules of daunomycin, each by a methylene, in the spectra of adducts 3 and 4. Possibly, more than four adducts are present in these reactions because of the

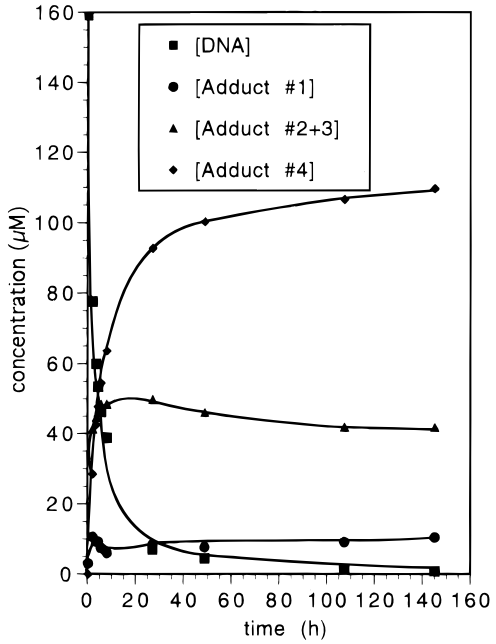


Figure 2. Concentration of DNA and DNA−daunomycin adducts 1−4 as a function of time for the reaction of $(GC)_4$ with daunomycin and formaldehyde in pH 7.0 phosphate buffer, starting with 168 μM $(GC)_4$, 337 μM daunomycin, and 337 μM formaldehyde.

abundance of formaldehyde. In fact, a shoulder was observed on the HPLC peak representing adduct 4.

The reactions of $(GC)_4$ with adriamycin and hydrogen peroxide in phosphate buffer (Table 1, entries 3 and 4) showed an additional DNA−drug adduct, designated 5. The mass spectrum of adduct 5 showed peaks for single-stranded DNA bound to one molecule of carboxylic acid **2** *via* a methylene group as shown in Figure 7 of Supporting Information. Both products of hydrogen peroxide oxidation of adriamycin, **2** and **3**, were also observed in the reaction mixture. Recall that formaldehyde is released upon formation of carboxylic acid **2**. In Tris buffer bearing iron ion, neither **2**, **3**, nor DNA−drug adduct 5 were observed. This result is interpreted in terms of Tris/iron, present in high concentration, diverting all of the hydrogen peroxide from the Baeyer−Villiger reaction to the Fenton oxidation of Tris. The corresponding reaction of $(GC)_4$ with daunomycin and hydrogen peroxide in phosphate buffer yielded almost no DNA−drug adducts. This is consistent with the lack of formaldehyde formation from Baeyer−Villiger oxidation of daunomycin.

Reversibility of Adduct Formation. As indicated above, attempts to isolate DNA−drug adducts can lead to decomposition back to starting DNA and drug. Similar decomposition of DNA−drug adducts was reported by Phillips and co-workers.[9,12] This observation suggested that the DNA−drug adducts might actually exist in equilibrium with each other and with free DNA and drug. Equilibration was established by performing the same reaction at two different states of dilution. One reaction mixture contained $(GC)_4$, daunomycin, and formaldehyde at 10 times higher concentrations than the other. Both reactions were maintained at 25 °C and monitored for DNA−drug adduct formation as a function of time. The results are shown in Figures 2 and 3. Both reactions appeared to approach equilibrium after about 150 h of reaction time. At equilibrium, the more concentrated reaction solution showed almost complete

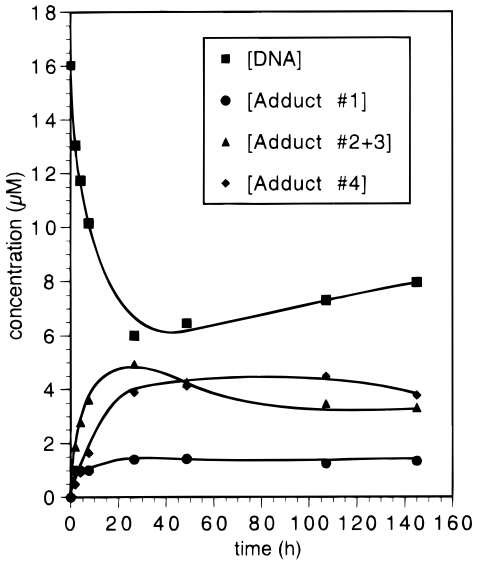


Figure 3. Concentration of DNA and DNA−daunomycin adducts 1−4 as a function of time for the reaction described in the legend to Figure 2 but with reagents at a 10-fold dilution.

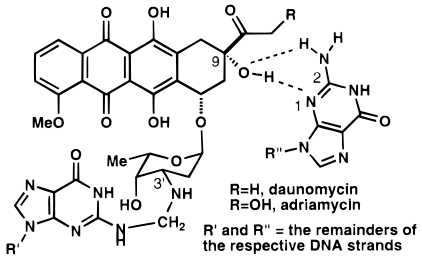
Table 3. Formation of Formaldehyde from Reaction of 5×10^{-4} M Spermine with 2.5×10^{-2} M Hydrogen Peroxide in pH 7.4 0.10 M Phosphate Buffer as a Function of Time

	[formaldehyde] (μM) at time		
buffer	17 h	40 h	4 days
PO_4^{3-}	<1	<1	2.3
PO_4^{3-}/EDTA (0.15 mM)	<1	<1	<1
PO_4^{3-}/Fe^{2+} (50 μM)	1.0	1.5	3.8
PO_4^{3-}/EDTA (0.15 mM)/ Fe^{2+} (50 μM)	2.4	16	32

reaction of the $(GC)_4$ with DNA−drug adduct 4 as the predominant product by a wide margin. The more dilute reaction mixture, however, showed almost one-half of the $(GC)_4$ remaining at equilibrium with adduct 4 approximately equal to the sum of adducts 2 + 3. Subsequent 10-fold dilution of the more concentrated reaction mixture ultimately gave a mixture of DNA and DNA−drug adducts similar to that formed in the original dilute reaction mixture. Although adducts 2 and 3 could not be completely resolved chromatographically, the relative abundance of each could be observed qualitatively. At the early stages of the reaction at high concentration, adduct 2 exceeded adduct 3; however, at the later stages adduct 3 exceeded adduct 2. Further, the ratio of adduct 2 to adduct 3 after 150 h of reaction was higher for the more dilute reaction. These observations are consistent with adduct 2 bearing one daunomycin and adduct 3 bearing two daunomycins. Reversibility of adduct formation is easily accommodated by the 1,1-diaminomethane linkage.

Formaldehyde from Spermine. Because Tris is not a natural buffer *in vivo*, production of formaldehyde from its Fenton oxidation is not a biological pathway to DNA alkylation. However, a number of amines are present in the cell, even in proximity to DNA. One example is the polyamine spermine. Reaction of spermine with hydrogen peroxide in phosphate buffer also produces formaldehyde, especially in the presence of iron and EDTA as shown in Table 3. The amount of formaldehyde produced was substantially less than that observed with Tris; however, the concentration of spermine was approximately 100-fold lower than the concentration of Tris in buffer.

Chart 1



Discussion

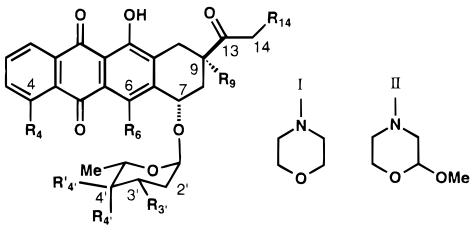
The results of our experiments indicate that adriamycin and daunomycin form similar covalent adducts with DNA from reaction with three dramatically different reagents: DTT, hydrogen peroxide, and formaldehyde. The species responsible for creating the covalent bond between DNA and drug in all cases is formaldehyde. The proposed adduct structures have one or two drugs intercalated in the DNA, each covalently bound to the DNA. The covalent attachment involves a methylene group from formaldehyde linking the 2-amino group of a G base to the 3′-amino group of the drug.

The DNA−drug adducts generated with DTT were produced in the same manner as described by Phillips and co-workers, and the UV−vis spectra and reactivity patterns are very similar. Perfect comparison is not possible because the DNAs were substantially different; Phillips employed much longer sequences. We selected a short sequence for which mass spectral characterization would be possible. Phillips and co-workers emphasized the importance of iron for achieving high adduct yields and reported that the adducts were labile with respect to isolation and redissolution in fresh buffer. They described two types of adducts in terms of stability, one longer lived and one shorter lived, and proposed that the longer lived adduct might actually be a "cross-link" at 5′-GpC-3′ sites and the shorter lived one an adduct at isolated dG residues. They proposed that the "cross-link" has two covalent bonds, one to each dG on opposing strands at the 2-amino substituents. The Wang X-ray structure for double-stranded $(CG)_3$ bound to two molecules of daunomycin with formaldehyde, shown schematically in Scheme 3, provides an explanation for a longer lived adduct at a 5′-GpC-3′ site without formation of an actual "cross-link". The X-ray structure shows hydrogen bonding between the 9-hydroxyl group of daunomycin and the nitrogens at the 1- and 2-positions of the dG on the strand opposing the formaldehyde covalent bond, as shown in Chart 1. Such hydrogen bonding would not be possible if the site were other than 5′-GpC-3′. We offer the descriptor *virtual cross-link* for this overall structure involving a covalent bond to one strand, strong hydrogen bonding to the other strand, and intercalation between both strands. We propose that Phillips' longer lived adduct is in fact this *virtual cross-link*.

Phillips and co-workers also tested a number of anthracycline derivatives for their capacity to form adducts.[21] Of particular interest were the derivatives which did not form adducts: 6-*O*-methyldaunomycin (**6**), 9-*O*-methyldaunomycin (**7**), 4-demethoxy-3′-deamino-4′-deoxy-4′epiaminodaunomycin (**8**), 4-demethoxy-3′-deam-

Chart 2

Anthracycline derivatives which do not form DNA adducts upon reductive activation



structure	difference from daunomycin or adriamycin
6	R_6 = OMe
7	R_9 = OMe
8	R_4 = H; $R_{3'}$ = H; $R_{4'}$ = H; $R'_{4'}$ = NH_2
9	R_4 = H; $R_{3'}$ = OH; $R_{4'}$ = H; $R'_{4'}$ = OH
10	$R_{3'}$ = morpholinyl structure I
11	$R_{3'}$ = morpholinyl structure II

ino-3′-hydroxy-4′-deoxy-4′-epiaminodaunomycin (**9**), 3′-deamino-3′-(4-morpholinyl)adriamycin (**10**), and 3′-deamino-3′-(2-methoxy-4-morpholinyl)adriamycin (**11**) as shown in Chart 2. Most of these differ with respect to the critical 3′-amino group: the 3′-amino is missing, epimerized, or alkylated. All of these modifications would make DNA adduct formation at 5′-GpC-3′ difficult or impossible with formaldehyde. In derivative **7** the important hydrogen bonding 9-OH is blocked by alkylation. In derivative **6**, only the 6-hydroxyl substituent is alkylated; possibly this transformation affects intercalation. Not among those derivatives studied by Phillips and co-workers but of relevance to the covalent bond-forming mechanism described here are the *N*-acylanthracyclines. Acetylation, but not trifluoroacetylation, of the amino group makes the anthracycline less active.[22] Possibly, the acetamide is more inert with respect to hydrolysis to the free amine.

Phillips and co-workers also reported that xanthine oxidase/NADH catalyzes DNA alkylation.[9,21] These experiments were similarly performed in transcription buffer containing a high concentration of Tris. A natural component of commercial xanthine oxidase preparations is iron. Hence, this reducing mixture also has the capacity to produce formaldehyde from Tris buffer.

Could the covalent linkage of adriamycin or daunomycin to DNA *via* formaldehyde Schiff base chemistry be an important cytotoxic event? Skladanowski and Konopa have detected the formation of unstable DNA cross-links in tumor cells induced by anthracyclines and have reported a strong correlation between cytotoxicity and cross-linking.[23,24] Certainly the oxidation of Tris to formaldehyde *via* Fenton chemistry is not an *in vivo* pathway to formaldehyde. However, we demonstrated that formaldehyde is also formed from Fenton oxidation of a biologically available polyamine, spermine. Spermine was chosen because it resides near DNA; hence, its oxidation catalyzed by adriamycin or daunomycin would produce the formaldehyde near the target. The Fenton reaction requires a redox active metal catalyst such as iron or copper. Adriamycin and daunomycin are excellent chelators of iron[25,26] and, consequently, might help locate iron at the reactive site. Although $(GC)_4$ used by us and $(CG)_3$ used by Wang and co-workers are special DNA sequences, natural nucleic acids are clearly also alkylated. Phillips and co-workers employed calf thymus DNA among other DNAs in their studies,[9] and Chaires and co-workers, in their extension of the Wang experiment, employed plasmid pBR322 DNA.[27] At the time of publication, Chaires and co-workers did not recognize the relationship between their work and the work of Phillips and co-workers.

Could the formaldehyde coupling reaction be a source of differential cytotoxicity? A few reports indicate that tumor cells have higher levels of formaldehyde than corresponding normal cells. Some years ago Thorndike and Beck reported that lymphocytic leukemia cells have higher levels of formaldehyde than normal lymphocytes.[28] Ebeler and co-workers have observed higher levels of formaldehyde in tumor-bearing, transgenic mice and cancer patients.[29] Bagachi and co-workers have found higher levels of formaldehyde in the urine of mice treated with adriamycin.[30]

The formaldehyde-mediated DNA alkylation reaction may also be involved in the carcinogenicity associated with adriamycin and daunomycin. Both drugs have been shown to induce mammary adenocarcinomas in female Sprague−Dawley rats.[31] Purewal and Liehr have demonstrated covalent DNA adduct formation in rat liver DNA incubated with daunomycin, rat liver microsomes and nicotinamide adenine dinucleotide phosphate (NADPH).[32] Even a higher level of DNA adduct formation was observed with cumene hydroperoxide serving as a cofactor. These reaction conditions would likely induce the production of formaldehyde.

Increased tumor cell response to doxorubicin combined with a reducing agent has been reported *in vitro* and *in vivo*. Bartoszek and Wolf reported increased cytotoxicity to MCF-7 human breast cancer cells upon addition of cytochrome P450 reductase and NADPH to the cell culture medium.[33] We reported increased survival of tumor-bearing mice treated with doxorubicin and a nontoxic, chemical reducing agent.[34]

Several natural and unnatural derivatives of adriamycin and daunomycin, which have the potential for covalent bond formation to DNA built into their structure, have been characterized. Four of these, (cyanomorpholino)doxorubicin,[35] barminomycin,[36,37] 2-pyrrolinodoxorubicin,[38] and *N*-(5,5-diacetoxypentyl)doxorubicin,[39,40] are shown in Chart 3. Each bears a masked aldehyde which could form covalent linkages to amino substituents of DNA, and each is significantly more cytotoxic than adriamycin or daunomycin. Increased cytotoxicity, in of itself, is not the answer to a better antitumor drug. Enhanced differential cytotoxicity to both sensitive and resistant tumor cells is the desirable property. Possibly, the lability of the adriamycin or daunomycin formaldehyde DNA adducts is important for differential cytoxicity, at least to sensitive cells.

Conclusions

The steps from drug reduction to DNA−drug adduct formation are summarized in Scheme 4. The process involves a cascade of reactions starting with drug reduction and catalytic production of reactive oxygen species followed by oxidative synthesis of formaldehyde and ending with drug−DNA alklyation mediated by formaldehyde. Alternative sources of formaldehyde not involving the redox chemistry of the drug are also possible. The results of this investigation answer a few

Scheme 4

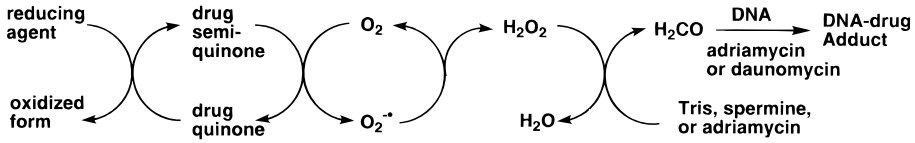
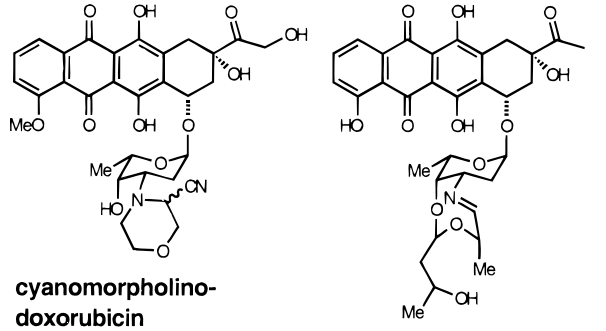
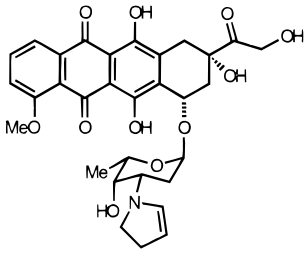
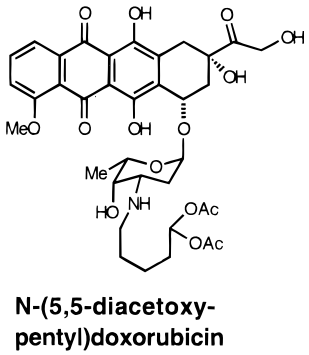


Chart 3







questions and raise more. At present we do not know the order of steps for the actual formaldehyde−DNA coupling reaction. Does the formaldehyde form a covalent bond first to the drug or to the DNA? Does the drug intercalate into the DNA before it reacts with formaldehyde? Correspondingly, we do not know the order of steps for the hydrolysis of the DNA−drug adducts. Does DNA sequence provide additional specificity? The model presented here, and by Wang and co-workers earlier, utilized a three-nucleotide sequence (5′-CpGpC-3′) with the CpG portion for intercalation and the GpC portion for hydrogen bonding and covalent attachment. The results of Phillips and co-workers suggest that only the GpC portion is critical. The nature of alkylation at isolated dG residues may be structurally related, only lacking the hydrogen-bonding interaction to the dG on the opposing strand. Does DNA alkylation *via* formaldehyde play a role in formation of topoisomerase-mediated DNA lesions? The answers to these and other questions will be the object of future work.

Experimental Details

General. UV−vis spectra were recorded with a Hewlett-Packard 8452A diode array spectrometer, DNA melting temperatures with a Varian Cary 1 spectrometer equipped with a Varian temperature control accessory and ^{1}H NMR spectra with a Bruker Model AM-400 spectrometer. Mass spectrometry (MS) and collision-induced dissociations (MS/MS) were carried out on an API-III triple-quadrupole mass spectrometer (Sciex) equipped with a nebulization-assisted electrospray (ES) ion source and a high-pressure collision cell. Samples were introduced by direct infusion of water/methanol (75/25, v/v) solutions or by HPLC.

Adriamycin and daunomycin were received as gifts from Pharmacia-Upjohn, Inc., Milano, Italy, and Nexstar Pharmaceuticals, Inc., San Dimas, CA, and used as received. DNA oligonucleotides were obtained from Integrated DNA Technologies, Coralville, IA, and were used as received or further purified by reverse phase HPLC as indicated. Water was distilled and purified with a Millipore Q-UF Plus purification system to 18 MΩ cm. Water used in DNA reactions was autoclaved. Stock solutions of hydrogen peroxide were prepared from 30% hydrogen peroxide from Mallinckrodt, diluting with Millipore water. Dithiothreitol (DTT) was >99% from Sigma; triethylamine for preparation of triethylammonium acetate, HPLC buffer was 99$^+$% from Aldrich; spermine was 98% from Aldrich; ethylenediaminetetraacetic acid (EDTA) was from Mallinckrodt; tris(hydroxymethyl)aminomethane (Tris) was from Boehringer Mannheim.

HPLC analyses and small scale separations were performed with a Hewlett-Packard 1090 liquid chromatograph equipped with a diode array UV−vis detector and workstation. Chromatographies were performed with either a Hewlett-Packard 5 μm C_{18} microbore column, 2.1 mm i.d. × 10 cm, eluting at 0.5 mL/min or a Rainin 5 μm C_{18} column, 4.6 mm i.d. × 25 cm, eluting at 1.0 mL/min, both with detection at 260 and 480 nm. Some preparative separations were performed with a Rainin 3 μm C_{18} Microsorb column, 10 mm i.d. × 50 mm, eluting at 3 mL/min with detection at 260 nm using either the Hewlett-Packard HPLC or a Rainin semipreparative HPLC equipped with a Dynamax Model UV-1 detector. Analytical and preparative elutions were performed with gradients of pH 6 triethylammonium acetate (Et_3NHOAc) (0.02 M)/acetic acid (AcOH) buffer and acetonitrile; some preparative HPLC was also performed with a gradient of water and acetonitrile. Ratios of DNA-bearing constituents were determined by integration of HPLC peaks with detection at 260 nm and ratios of anthracycline constituents, at 480 nm, both without correction for differences in molar absorptivity unless otherwise indicated.

Analyses for formaldehyde were performed according to Nash by mixing equal volumes of the Hantzsch reagent and the solution containing formaldehyde and reacting at ambient temperature for 5 h or at 57 °C for 5−10 min before reading the absorbance at 412 nm.[16] The Hantzsch reagent was an aqueous solution 2 M in ammonium acetate, 0.05 M in acetic acid, and 0.02 M in 2,4-pentanedione. The identity of the yellow compound from reaction with the Hantzsch reagent was checked by HPLC comparison with a sample prepared with formalin; the retention time was 3.0 min eluting at 0.50 mL/min with 15% acetonitrile/85% triethylammonium acetate, pH 6, buffer; the UV spectrum showed peaks at λ_{max} 255, 282 (sh), 425 (rel intensity, 1:0.6:0.7).

Reaction Buffers. Buffer A = pH 7.4, 40 mM Tris/0.15 mM EDTA/100 mM KCl/3.2 mM $MgCl_2$. Buffer B = pH 8.0, 93.2 mM Na_2HPO_4/6.8 mM NaH_2PO_4. Buffer C = pH 7.0, 57.7 mM Na_2HPO_4/42.3 mM NaH_2PO_4. Buffer D = pH 8.0, 40 mM Tris/0.10 mM EDTA/100 mM KCl/3mM $MgCl_2$.

HPLC Elution Methods. The following HPLC elution gradients were employed with A = HPLC grade acetonitrile and B = triethylamine (20 mM)/acidified to pH 6 with acetic acid unless otherwise specified: HPLC I, 0.5 mL/min, A:B, 0:100 to 70:30 at 10 min, isocratic until 12 min, 70:30 to 0:100 at 15 min; HPLC II, 3.0 mL/min, B = HPLC grade H_2O, A:B, 0:100 to 70:30 at 10 min, isocratic until 12 min, 70:30 to 0:100 at 15 min; HPLC III, 0.5 mL/min, A:B, 0:100 to 7:93 at 1 min, to 9:91 at 10 min, to 70:30 at 13 min, isocratic until 15 min, to 0:100 at 17 min; HPLC IV, 0.5 mL/min, A:B, 0:100 until 1 min, to 10.5:89.5 at 2 min, isocratic until 15 min, to 15:85 at 18 min, to 70:30 at 22 min, isocratic until 23 min, to 0:100 at 25 min.

Purification of $(GC)_4$ and $(GC)_4$−Anthracycline Adducts. Commercially prepared $(GC)_4$ was collected from a Rainin 3 μm C_{18} reverse phase column (10 mm i.d. × 5 cm) using HPLC method III, eluting at 3.0 mL/min. The solvent was removed by vacuum centrifugation (0.05 Torr), and the DNA was stored at −20 °C until needed. $(GC)_4$−anthracycline adducts were preparatively separated on a C_{18} analytical column, either the Hewlett-Packard microbore column or the Rainin 4.6 mm i.d. × 25 cm column, eluting with method III or IV. In addition to the four normal $(GC)_4$−anthracycline adduct peaks described below, additional small peaks were observed in the region 11.7−11.9 min. The intensity of these latter peaks was variable. They appeared to represent that portion of the DNA and DNA−anthracycline adducts which for some unknown reason immobilized at the head of the column and eluted only at high acetonitrile concentration. The mass spectrum of this material was similar to the mass spectra of the normal DNA−anthracycline adducts, except when carboxylic acid **2** was present (vide infra).

Melting Temperature of $(GC)_4$. The melting temperatures of $(GC)_4$ in buffer A and in 90% HPLC buffer/10% acetonitrile were determined from melting curves with 20 μM $(GC)_4$ ramping the temperature from 5 to 93 °C at a rate of 1 °C/min. The composition of the HPLC eluent at the point in the gradient where the DNA and DNA−drug adducts eluted was estimated to be 90% buffer/10% acetonitrile. The melting temperatures were 61 °C in buffer A and 45 °C in 90% HPLC buffer−10% acetonitrile.

Removal of Buffer from HPLC-Collected DNA Adducts Prior to ESMS Analysis. The following procedure was required because of the instability of the anthracycline−DNA adducts and ESMS noise from a large excess of buffer in samples for mass spectral analysis with direct infusion. Approximately 30% (v/v) of 0.1 M sodium phosphate buffer (pH 7) was added to each of the collected HPLC fractions. The solvent was then removed by vacuum centrifugation (0.05 Torr). The dry product/phosphate solid remaining was resuspended in 50 μL of Millipore water (autoclaved), and the adducts were recollected by preparative HPLC. The method for the second collection utilized acetonitrile (A) and Millipore water (B): A:B, isocratic 0:100 for 3 min, then to 70:30 at 7 min, returning to 0:100 at 10 min; DNA adducts eluted at approximately 5 min. This effectively removed the phosphate salts from the DNA. Solvent was removed from the adducts by vacuum centrifugation (0.05 Torr), and the dry adducts were stored at −20 °C until ESMS analysis. Recollection to remove phosphate salts was not performed when LC/ESMS analyses were utilized.

HPLC Electrospray Mass Spectrometry. For HPLC/ESMS the column consisted of a glass capillary (0.5 mm i.d. × 15 cm length packed with Vydac C12; the column was eluted with Millipore-purified water at 20 μL/min for 10 min to remove salts and then with 50% HPLC grade acetonitrile/50% Millipore water (v/v) at 20 μL/min for 10 min. The DNA−drug adducts entered the mass spectrometer after about 2 min of elution with acetonitrile/water. Once all of the sample was eluted, the 200 μL column loop was rinsed with 2 × 200 μL of water, and the column was eluted with water at 20 μL/min for 6 min prior to the next injection. Immediately prior to injection, the sample loop was filled with 1 mM triethylamine to reduce the size of potassium adduct peaks.

(7*S*,9*S*)-7-[(3-Amino-2,3,6-trideoxy-α-L-*lyxo*-hexopyranosyl)oxy]-9-carboxy-7,8,9,10-tetrahydro-6,9,11-trihydroxy-4-methoxy-5,12-naphthacenedione (2) and 7,8-Dehydro-9,10-desacetyldaunorubicinone (3) from Reaction of Adriamycin with Hydrogen Peroxide. To 35 mL of 0.1 M sodium phosphate buffer (buffer B, pH 8) were added 6 mg of adriamycin (10.3 μmol) and 160 μL of a 9.8 M hydrogen peroxide (H_2O_2) solution (157 μmol). The mixture was allowed to react for 4 days in the dark at ambient temperature. At that point the adriamycin was ∼95% converted to **2** and **3**, which were largely present as precipitates. The solvent was removed by high-vacuum rotary evaporation (0.05 Torr), and the remaining solid was dissolved in methanol for HPLC analysis (Rainin 3 μM C_{18} column, method II), which showed 23% **2** with retention time 5.9 min and 73% **3** with retention time 11.9 min after correction of peak areas for differences in molar extinction coefficients at 260 nm. The molar extinction coefficient for **2** was assumed to be 26 100 M^{-1} cm^{-1}, as reported earlier at 252 nm,[14] and for **3**, 14 700 M^{-1} cm^{-1}, as determined here in 4:1 methanol/water (v/v) at 260 nm. The product mixture was separated by preparative HPLC (method HPLC II), and the elution solvent was removed from the fractions by vacuum centrifugation (0.05 Torr). ^{1}H NMR analysis of the material presumed to be product **3** showed that the HPLC method transformed the product into a new material which has not been identified. Dry column chromatography on silica gel ultimately provided a pure sample of **3** for spectral comparison with literature data (vide infra). Comparison of product **2** with an authentic sample (vide infra) by HPLC, ^{1}H NMR, and UV−vis absorption established identity. Electrospray mass spectral analysis of **2** gave the following data: MS/MS, positive ion, *m*/*z* (relative intensity) 552.1 (100, $M + Na^+$, calcd for $C_{26}H_{27}NO_{11}Na$ 552.1), 441 (8), 423 (92), 405 (92), 387 (12), 343 (8), 170 (17), 152 (8).

Formation of 2 and 3 from Reaction of Adriamycin with Dithiothreitol (DTT). To a 0.508 mM solution of adriamycin (10.2 μmol in 20 mL of pH 8 12.4 mM Tris/1.0 mM EDTA/100 mM KCl buffer) was added 25 μmol of DTT (25 μL of a 1 M solution). The adriamycin buffer solution was sonicated prior to addition of DTT to effect solubilization. The reaction mixture was kept in the dark at 37 °C for a total of 16 days; however, the composition changed little after 5 days as indicated by HPLC analysis. The precipitate which formed during this time was then collected by suction filtration and washed with 2 × 2 mL portions of Millipore water. The precipitate was dissolved in methanol and analyzed by HPLC (method I). The precipitate contained 3% **2** (4.89 min), 15% adriamycin (6.15 min), and 51% **3** (9.45 min). Other products were present which have not been characterized. Percentages are based upon peak areas monitoring at 480 nm and are uncorrected for modest differences in molar extinction coefficients at this wavelength.

Large Scale Reaction of Adriamycin with Hydrogen Peroxide (H_2O_2). A 4.93 mM solution of adriamycin was prepared by dissolving 24.75 mg (42.7 μmol) of adriamycin in a mixture of 4 mL of pH 7.4 Tris buffer (buffer A) and 4 mL of methanol. An excess of H_2O_2 was then added (654 μL of a 9.8 M solution, 150 equiv), and the mixture was allowed to react in the dark at ambient temperature for 14 days. The reaction mixture was then centrifuged for 20 min, and the supernatant liquid was removed. HPLC analysis showed the supernatant to contain a small amount of **2**, adriamycin, **3**, and other very minor products. The remaining precipitate was washed by suspension in 3 mL of Millipore water followed by centrifugation (1550 rpm). The washed precipitate was dried by suction filtration in a medium frit glass filter and determined to be 94% pure **3** by HPLC (method I, monitoring at 260 nm). Product **3** was characterized by comparison of its ^{1}H NMR spectrum with a literature spectrum[15] and further identified by electrospray mass spectrometry: ^{1}H NMR (DMSO-d_6) δ 3.96 (s, OMe, 4), 7.31 (dd, J = 3, 8 Hz, 8), 7.49 (d, J = 8 Hz, 7), 7.60 (d, J = 3 Hz, 10), 7.84 (t, J = 8.5 Hz, 2), 7.96 (d, J = 8 Hz, 3), 8.22 (d, J = 9 Hz, 1), 12.7 (s, OH, 9); ESMS, negative ion, *m*/*z* (rel intensity) 335 (100, M − 1), 255 (33), 249 (18), 241 (8).

Reaction of Adriamycin with H_2O_2 in Phosphate Buffer with Detection of Formaldehyde. A 10 mL solution of adriamycin (133 μM) and hydrogen peroxide (16.0 mM) in pH 8.0 phosphate buffer (buffer B) was allowed to react in the dark at 24 °C for 6 days. At this time, 70% of the adriamycin had been converted to the carboxylic acid **2** and 8% to the aglycon **3** as determined by HPLC (method I) with detection at 480 nm; 15% of the adriamycin was still present. The reaction mixture was analyzed for the presence of formaldehyde using the Hantzsch reagent together with HPLC analysis as described under General remarks. The concentration of formaldehyde present was determined to be 6 μM (6% based upon formation of **2**) from the HPLC peak area and a calibration curve prepared from standard solutions of formaldehyde. The low yield of formaldehyde resulted from instability of formaldehyde to the reaction conditions as indicated by the following control experiment. A 10 mL solution of buffer B containing 106 μM formaldehyde and 16

mM hydrogen peroxide was allowed to react in the dark at 25 °C. Hantzsch analysis for formaldehyde showed a 91% decrease in formaldehyde concentration after 24 h and a 94% decrease after 4 days. A second control experiment containing only 106 μM formaldehyde in buffer B showed a 37% decrease in formaldehyde concentration after 24 h with no further change over 4 days. Hydrogen peroxide oxidation of formaldehyde to formate is precedented.[41]

Reaction of Daunomycin with H_2O_2. A 2.0 mL solution of pH 7.4 Tris buffer (buffer A) containing 84.5 mg (0.15 mmol) of daunomycin was added to 1.0 mL of 9.8 M H_2O_2 (9.8 mmol, 65 equiv). The mixture was kept in the dark at ambient temperature for 7 days. HPLC analysis (method I) revealed the mixture to contain 41% **3** (retention time 9.3 min) and 33% of a single unidentified product (retention time 3.8 min) with the balance distributed between other unidentified products based upon peak areas monitoring at 480 nm. The reaction mixture was then filtered through a medium frit glass filter funnel and the solid washed with 3 × 5 mL of pH 7.4 Tris buffer followed by 3 × 5 mL of Millipore water. The filtrates contained 60% of the single unidentified product and less than 10% of **3**. The precipitate (15.4 mg) was further purified by dry column silica gel chromatography. The material was dissolved in a small amount of methanol and loaded onto a 15 mL medium frit Buchner filter funnel packed with dry silica gel (40−63 μm). Solvent elution was as follows: 12 mL of 14:1 methylene chloride (CH_2Cl_2)/propanol, 12 mL of 4:1 CH_2Cl_2/propanol, 16 mL of 2:1 CH_2Cl_2/propanol, 12 mL of 2:1 propanol/water, and 16 mL of 1:1 propanol/water (v/v). Product **3** appeared at the end of the elution with 4:1 CH_2Cl_2/propanol through the elution with 2:1 CH_2Cl_2/propanol. The combined fractions were ∼87% pure **3**, based upon HPLC analysis (method II, monitoring at 260 nm). The ^{1}H NMR spectrum as well as the HPLC retention time and UV−vis absorption matched samples of **3** obtained from reaction of adriamycin with H_2O_2 or DTT.

Reaction of Adriamycin with Sodium Periodate ($NaIO_4$). The procedure was adapted from a literature procedure.[14] An 8.6 mg sample (14.8 μmol) of adriamycin was dissolved in 900 μL of 2:1 water/methanol to which was added 148 μL of a 0.1 M $NaIO_4$ (14.8 μmol) aqueous solution. The mixture was placed in an ice bath in the dark for 2 h. The solution (pH 2.8) was then adjusted to pH 5.8 by the addition of 84 μL of a 0.20 M sodium carbonate solution. The gelatinous suspension was then kept in an ice bath with periodic stirring for 45 min. The precipitate was then isolated by centrifugation and washed with 2 × 300 μL of Millipore water followed by centrifugation. In each case centrifugation lasted 20 min and was at 1550 rpm. After the second wash, the precipitate was transferred to a 15 mL medium frit filter funnel and gravity filtered overnight in the dark. The sample was then further dried by suction filtration for 60 min. HPLC analysis of the precipitate (method I) showed the material to be greater than 95% pure **2**. The filtrates from the water washes were also analyzed by HPLC and shown to contain greater than 85% **2** relative to other species with absorption at 480 nm. The precipitate dissolved in methanol-d_4 gave the following ^{1}H NMR spectrum: δ 1.22 (d, J = 6 Hz, 5′-Me), 1.80 (dd, J = 5, 13 Hz, 2′), 1.88 (td, J = 13, 4 Hz, 2′), 2.31 (dd, J = 2, 15 Hz, 8), 2.36 (dd, J = 5, 15 Hz, 8), 3.09 (br s, 10), ∼3.3 (m, buried under the solvent signal, 3′), 3.54 (m, 4′), 4.04 (s, 4-OMe), 4.32 (q, J = 6 Hz, 5′), 5.06 (dd, J = 2, 5 Hz, 7), 5.41, (br d, J = 4 Hz, 1′), 7.57 (br d, J = 8 Hz, 1), 7.84 (t, J = 8 Hz, 2), 7.98 (br d, J = 8 Hz, 3). Attempts to obtain the ^{1}H NMR spectrum of **2** or its hydrochloride salt in DMSO-d_6 showed the material to be unstable in DMSO. The free base degraded completely in 2 h and the hydrochloride salt, 30% in 2 h.

Reaction of $(GC)_4$ with Adriamycin and Dithiothreitol in pH 8 Tris Buffer Containing Ferric Chloride ($FeCl_3$). A 1 mL solution of pH 8 Tris buffer (buffer D) containing 18 μM HPLC-purified $(GC)_4$, 37 μM adriamycin, 7 mM DTT, and 40 μM $FeCl_3$ was allowed to react in the dark at 25 °C for 4 days. At this time the DNA had been completely converted to DNA−drug adducts. HPLC (method III) showed four DNA−drug peaks, 1−4, appearing at 7.4, 8.3, 8.6, and 9.7 min, respectively (DNA appeared at 6.9 min). Peaks 2 and 3 were poorly resolved. See Figure 4 of Supporting Information for the actual chromatogram. The area ratio of unreacted DNA: 1:(2 + 3):4 was 0:19:35:45. UV−vis spectra of the materials represented by the HPLC peaks, comparing absorption at 480 nm to absorption at 260 nm, indicated that 1 and 2 represented mono-drug−DNA adducts (absorbance ratio ∼1:20) and 3 and 4, bis-drug−DNA adducts (absorbance ratio ∼1:10). A larger scale reaction with 37 nmol of $(GC)_4$ and 148 nmol of adriamycin was subsequently performed. The products were separated by preparative HPLC as described above and analyzed by ESMS. The mass spectra showed the products to be drug−DNA adducts with the drug attached *via* a methylene group as shown in Figure 5 of Supporting Information.

Reaction of $(GC)_4$ with Adriamycin and Dithiothreitol in pH 8 Tris Buffer Containing Ferric Chloride ($FeCl_3$) in the Presence and Absence of Molecular Oxygen. Two identical reaction mixtures were run in parallel. Each mixture consisted of 1 mL of pH 8 Tris buffer (buffer D) containing 24 μM HPLC-purified $(GC)_4$, 70.8 μM adriamycin, 7 mM DTT, and 40 μM $FeCl_3$. One reaction mixture was freeze (77 K)−thaw-pump (7×10^{-6} Torr)-degassed through three cycles; after the last cycle the vessel was sealed with a torch simultaneously with evacuation at 7×10^{-6} Torr. The second reaction mixture was maintained in contact with air. Both reaction mixtures were kept in the dark at ambient temperature for 4 days. At this time the sealed vessel was cracked open and the reaction mixture immediately analyzed by HPLC. HPLC analysis (method III) showed that none of the DNA in the degassed reaction mixture had reacted and that all of the DNA in the reaction mixture exposed to air had reacted to give the mixture of drug−DNA adducts described above. After exposure to air the degassed reaction mixture ultimately produced the drug−DNA adducts.

Reaction of $(GC)_4$ with Adriamycin and Hydrogen Peroxide in pH 7.4 Tris Buffer. A 650 μL solution of pH 7.4 Tris buffer (buffer A) containing 104 μM $(GC)_4$, 155 μM adriamycin, and 3.1 mM hydrogen peroxide was allowed to react in the dark at 25 °C for 4 days. At this time 60% of the DNA and 56% of the adriamycin were converted to products based upon HPLC analysis detecting at 260 nm (method III). The area ratio of unreacted DNA to drug−DNA adducts represented by peaks 1, 2 + 3, and 4 was 40:5:20:35. The products were collected from the HPLC as described earlier except they were not collected a second time to remove salts; instead they were analyzed by LC/ESMS. The mass spectra showed that all of the products contained adriamycin covalently bound to DNA *via* a methylene group. The major product represented by peak 4 showed one adriamycin bound to each strand of DNA *via* a methylene group as shown in Figure 1.

Reaction of $(GC)_4$ with Adriamycin in pH 8 Phosphate Buffer in the Presence of Hydrogen Peroxide. A 250 μL solution of pH 8.0 phosphate buffer (buffer B) containing 134 μM $(GC)_4$, 270 μM adriamycin, 2.7 mM hydrogen peroxide, and 1.6 mM $MgCl_2$ was allowed to react for 6 days in the dark at ambient temperature. At this time, very few products were present, and an additional 670 nmol of H_2O_2 was added (6.7 μL of a 0.1 M solution). After 8 more days (14 days total), 17% of the DNA and 47% of the adriamycin had reacted as indicated by HPLC (method III). Anthracycline products **2** and **3** were also present, but their precipitation precluded accurate quantitation. The DNA products were represented in the HPLC primarily by the same four peaks as described for the reaction in Tris buffer; the area ratio of unreacted DNA:1:(2 + 3):4 was 83:8:6:1. Four of these reactions were run in parallel to provide enough material for ESMS analysis. The DNA products were then separated by HPLC (method III), and buffer was removed as described earlier. Subsequent ESMS analysis revealed the products to contain the DNA with adriamycin covalently bound by a methylene linkage. The MS of an additional DNA product represented by a peak at longer retention time (peak 5, 11.7 min; DNA:5 = 83:2) showed carboxylic acid **2** bound to the DNA *via* a methylene group as shown in Figure 7 in Supporting Information. A similar experiment was also performed in pH 7.0 phosphate buffer (buffer C) with all of the hydrogen peroxide present at time zero. After 12 days of reaction the ratio of DNA- bearing constituents, DNA:1:(2 + 3):4:5, was 60:11:16:5:8.

Reaction of $(GC)_4$ with Daunomycin in Tris Buffer in the Presence of Hydrogen Peroxide and EDTA. A 0.34 mM solution of $(GC)_4$ was prepared by dissolving 0.10 mg of $(GC)_4$ in 0.10 mL of a 0.43 mM solution of daunomycin in pH 8.0 Tris buffer (buffer D) containing 4.0 mM hydrogen peroxide. After 1 day at ambient temperature the HPLC profile (method IV), monitoring at 260 nm, showed three peaks in a 1:2:0.5 ratio with retention times 6.4, ca. 7.2, and 8.3 min, accounting for ca. 20% of the $(GC)_4$ peak at 5.7 min. The 7.2 min peak was a result of the partial overlap of two peaks separated by 0.2 min. According to the increasing retention times, the products and peaks were called 1, 2, partially overlapped to 3, and 4. The UV spectra showed an A_{260}/A_{480} ratio of 20 for peaks 1 and 2, and of 10 for the peaks 3 and 4. After a total of 3 days the 1:(2 + 3):4 ratio, now accounting for 60% of the total area, was 1:3:2. At 9 days $(GC)_4$ had almost completely reacted, and the 1:(2 + 3):4 ratio was 1:3:4. A similar result was obtained when the reaction was run in pH 7.4 Tris buffer (buffer A).

In the absence of EDTA at pH 7.4, even using 30 mol equiv of hydrogen peroxide, the reaction was much slower, with <2% conversion of $(GC)_4$ in 2 days. Addition of Fe^{2+} (5×10^{-5} M ferrous sulfate, $FeSO_4$) made the reaction moderately faster (ca. 10% of peaks 1−3 in 2 days). The simultaneous addition of 5×10^{-5} M Fe^{2+} and 1.5×10^{-4} EDTA made the reaction much faster, with 40% conversion in 1 day. However, part of $(GC)_4$ appeared to be degraded, as shown from the appearance of a set of broad peaks with retention times 2−4 min, showing no anthracycline absorbance.

Isolation of the Major Product from the Reaction of $(GC)_4$ with Daunomycin in the Presence of Hydrogen Peroxide in Tris Buffer. A reaction was run for 5 days as described above starting from 0.2 mg of $(GC)_4$, at pH 7.4 (buffer A). The constituents were preparatively separated using HPLC method III and a Hewlett-Packard microbore C_{18} column, eluting at 0.5 mL/min. Injections were 10−30 μL in volume. When part of the combined peak 4 (9 min) fractions was reinjected, the peak appeared unchanged. However, when the fractions were concentrated by vacuum centrifugation, reinjection of the residue showed extensive decomposition to give mostly $(GC)_4$ and daunomycin. To prevent such decomposition, 20% (v/v) of 0.1 M pH 7.0 phosphate buffer was added to the combined peak 4 fractions prior to centrifugal vacuum concentration. With this artifice, peak 4 was collected and the solvent removed by vacuum centrifugation with no decomposition. ESMS analysis produced the spectrum shown in Figure 1 of the earlier communication on this subject.[13]

Reaction of $(GC)_4$ with Daunomycin and Formaldehyde in Tris Buffer. A 0.34 mM solution of $(GC)_4$ was prepared by dissolving 0.50 mg of $(GC)_4$ in 0.50 mL of a 0.41 mM solution of daunomycin in pH 7.4 Tris buffer (buffer A minus the EDTA) containing 1.7 mM formaldehyde. After 1 h the HPLC profile (method III) showed a 40% conversion of the $(GC)_4$ peak (at retention time 5.5 min) into peaks 1, 2 + 3, and 4 in a 1:4:2 ratio, with retention times 6.5, 7.5 (2 + 3), and 8.7 min, respectively. The 7.5 min peak was the sum of two very close peaks (2 and 3), as more clearly suggested by the HPLC profile from detection at 480 nm. The UV spectra showed an A_{260}/A_{480} ratio of ca. 20 for peaks 1 and 2, and of ca. 10 for peaks 3 and 4. Coinjection with the reaction mixture from the reaction between $(GC)_4$ and daunomycin in the presence of hydrogen peroxide, as reported above, showed the identity of the two sets of four peaks. After 6 h no $(GC)_4$ was detected by HPLC. At this time the major peaks were 3 and 4, with peak 4 (at 8.7 min) showing a shoulder (4′) at ca. 9 min. Preparative HPLC was performed using the method IV and a 4.6 mm i.d. × 25 cm, Rainin column bearing 5 μm spherical C_{18} packing, eluting at 1.0 mL/min, with 50−100 μL injections. After the peaks were collected, 30% (v/v) of 0.1 M phosphate buffer was added to each of the combined fractions before centrifugal vacuum evaporation for electrospray mass spectrometry. ESMS analysis showed the products represented by peaks 1, 2 + 3, and 4 to be drug−DNA adducts with the drug attached *via* a methylene group as shown in Figure 6 of Supporting Information.

The rate of the reaction of $(GC)_4$ with daunomycin and formaldehyde depended on the concentration of the latter. In fact, when a reaction was run using 0.17 mM formaldehyde after 1/5 days only 10%/30% of $(GC)_4$ was converted into compounds 1−4. With 0.66 mM formaldehyde a 40% conversion was obtained in 1 day, with a 1:(2 + 3):4 ratio equal to 1:2:0.5.

Lack of Reaction of $(GC)_4$ with Daunomycin and Hydrogen Peroxide in pH 7 Phosphate Buffer. A 150 μL solution of pH 7 phosphate buffer (buffer C) containing 180 μM $(GC)_4$, 307 μM daunomycin, 3.96 mM H_2O_2, and 1.62 mM $MgCl_2$ was allowed to react in the dark at ambient temperature. After 12 days, HPLC analysis (method III) showed that the DNA was 95% unreacted. A control reaction without H_2O_2 showed 98% unreacted DNA.

Reaction of $(GC)_4$ with Daunomycin and Formaldehyde as a Function of Dilution. Two reactions, A and B, were run in parallel, each containing 16.8 nmol of $(GC)_4$, 33.7 nmol of daunomycin, and 33.7 nmol of formaldehyde in pH 7.0 phosphate buffer (buffer C). The total volume of the reaction mixtures was 100 μL for reaction A and 1000 μL for reaction B. This yielded $(GC)_4$, daunomycin, and formaldehyde concentrations of 168, 337, and 337 μM, respectively, for reaction A and 16.8, 33.7, and 33.7 μM, respectively, for reaction B. Each reaction mixture also contained 40 μM $FeCl_3$ and 3 mM $MgCl_2$. Both reaction vials were maintained in the dark at 25 °C and monitored by HPLC (method III) over a period of 145 h. Concentrations of $(GC)_4$ and $(GC)_4$−drug adducts were determined from peak areas with detection at 260 nm together with HPLC calibration data from injection of standard samples of $(GC)_4$. For the purpose of this semiquantitative experiment, the responses of $(GC)_4$ and all adducts were assumed to be identical. The results are shown in Figures 2 and 3. The same two reactions were performed a second time and allowed to proceed for 6 days. At this time HPLC analysis showed ratios of DNA:1:(2 + 3):4 analogous to those in Figures 2 and 3. The more concentrated reaction mixture A was then diluted 10-fold with buffer to establish the same concentration of reactants in both A and B. After an additional 7 days, reaction A equilibrated to a product mixture analogous to that of reaction B.

Reaction of $(GC)_4$ with Adriamycin and Formaldehyde in pH 7.4 Tris Buffer. A 500 μL solution of pH 7.4 Tris buffer (buffer A) containing 172 μM $(GC)_4$, 208 μM adriamycin, and 1.72 mM formaldehyde was allowed to react in the dark at 25 °C for 24 h. The DNA was completely converted to a mixture of the DNA−adriamycin adducts as indicated by HPLC (method III). The ratio of adduct 1:(2 + 3):4 was 8:30:62.

Formaldehyde from Oxidation of Tris by Hydrogen Peroxide. Six solutions (in vials A, B, C, D, E, F) were prepared, each containing 5.0 mL of 40 mM Tris buffer (pH 7.4 40 mM Tris/100 mM KCl/3 mM $MgCl_2$ buffer in vials A, C, and E; pH 8.0 40 mM Tris/0.10 mM EDTA/100 mM KCl/3 mM $MgCl_2$ buffer in vials B, D, and F). Ferrous sulfate was added to vials C and D to make $[Fe^{2+}] = 50$ μM, and ferric ammonium sulfate ($NH_4Fe(SO_4)_2$) was added to vials E and F to make $[Fe^{3+}] = 50$ μM. Hydrogen peroxide (10 μL of a 10 M solution) was added to each vial to make $[H_2O_2] = 20$ mM. The amount of formaldehyde obtained was measured using the method of Nash.[16] The results are reported in Table 2.

Formaldehyde from Oxidation of Spermine by Hydrogen Peroxide. A 0.50 mM solution of spermine was prepared in pH 7.4 0.10 M phosphate buffer. Four vials (A, B, C, D) were prepared, each containing 5.0 mL of the spermine solution. EDTA was added to vials B and D to make [EDTA] = 0.15 mM. Ferrous sulfate was added to vials C and D to make $[Fe^{2+}] = 50$ μM. Hydrogen peroxide (12.5 μL of a 10 M solution) was added to each vial to make $[H_2O_2] = 25$ mM. The amount of formaldehyde obtained was measured using the method of Nash.[16] The results are reported in Table 3.

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Supporting Information Available: HPLC chromatogram for reaction of $(GC)_4$ with adriamycin and DTT in pH 8.0 Tris buffer containing $FeCl_3$/EDTA (Figure 4); negative ion, electrospray mass spectra of the DNA−drug adducts from reaction of adriamycin with DTT in the presence of $(GC)_4$ in Tris buffer (Figure 5), of the DNA−drug adducts from reaction of daunomycin with formaldehyde in the presence of $(GC)_4$ (Figure 6), and of the additional DNA−drug adduct from reaction of adriamycin with H_2O_2 in the presence of $(GC)_4$ in phosphate buffer (Figure 7); table of calculated mass to charge ratios for ESMS ions (Table 4); proposed structures for mass spectral ions (Figure 8); and proposed schematic structures for DNA−drug adducts and mass spectral ions (Figure 9) (9 pages). Ordering information is given on any current masthead page.

References

(1) Young, R. C.; Ozols, R. F.; Myers, C. E. Medical Progress: The anthracycline antineoplastic drugs. *New Engl. J. Med.* **1981**, *305*, 139−153.

(2) Guaglianone, P.; Chan, K.; DelaFlor-Weiss, E.; Hanisch, R.; Jeffers, S.; Sharma, D.; Muggia, F. Phase I and pharmacologic study of liposomal daunorubicin (DaunoXome). *Invest. New Drugs* **1994**, *12*, 103−110.

(3) Liu, L. F. DNA topoisomerase poisons as antitumor drugs. *Annu. Rev. Biochem.* **1989**, *58*, 351−375.

(4) Pommier, Y. DNA topoisomerases and their inhibition by anthracyclines. In *Anthracycline Antibiotics: New Analogues, Methods of Delivery, and Mechanisms of Action*; Priebe, W., Ed.; ACS Symposium Series 574; American Chemical Society: Washington, DC, 1995; pp 183−203.

(5) Lown, W. J.; Chen, J. A.; Plambeck, J. A.; Acton, E. M. Further studies on the generation of reactive oxygen species from activated anthracyclines and the relationship to cytotoxic action and cardiotoxic effects. *Biochem. Pharmacol.* **1982**, *31*, 575−581.

(6) Doroshow, J. H. Role of reactive-oxygen metabolism in cardiac toxicity of anthracycline antibiotics. In *Anthracycline Antibiotics: New Analogues, Methods of Delivery, and Mechanisms of Action*; Priebe, W., Ed.; ACS Symposium Series 574, American Chemical Society: Washington, DC, 1995; pp 259−267.

(7) Gaudiano, G.; Koch, T. H. Redox chemistry of anthracycline antitumor drugs and use of captodative radicals as tools for its elucidation and control. *Chem. Res. Toxicol.* **1991**, *4*, 2−16.

(8) Egholm, M.; Koch, T. H. Coupling of the anthracycline antitumor drug menogaril to 2′-deoxyguanosine through reductive activation. *J. Am. Chem. Soc.* **1989**, *111*, 8291−8293.

(9) Cullinane, C.; van Rosmalen, A.; Phillips, D. R. Does adriamycin induce interstrand cross-links in DNA? *Biochemistry* **1994**, *33*, 4632−4638.

(10) Cutts, S. M.; Parsons, P. G.; Sturm, R. A.; Phillips, D. R. Adriamycin-induced DNA adducts inhibit the DNA interactions of transcription factors and RNA polymerase. *J. Biol. Chem.* **1996**, *271*, 5422−5429.

(11) Cutts, S. M.; Phillips, D. R. Use of oligonucleotides to define the site of interstrand cross-links induced by Adriamycin. *Nucleic Acids Res.* **1995**, *23*, 2450−2456.

(12) van Rosmalen, A.; Cullinane, C.; Cutts, S. M.; Phillips, D. R. Stability of adriamycin-induced DNA adducts and interstrand crosslinks. *Nucleic Acids Res.* **1995**, *23*, 42−50.

(13) Taatjes, D. J.; Gaudiano, G.; Resing, K.; Koch, T. H. Alkylation of DNA by the Anthracycline, Antitumor Drugs Adriamycin and Daunomycin. *J. Med. Chem.* **1996**, *39*, 4135−4138.

(14) Tong, G.; Lee, W. W.; Black, D. R.; Henry, D. W. Adriamycin Analogs. Periodate Oxidation of Adriamycin. *J. Med. Chem.* **1976**, *19*, 395−398.

(15) Tong, G.; Lee, W. W.; Black, D. R.; Garnier-Sullerot, A.; Amirand, C.; Ballini, J.-C.; Chinsky, L.; Duquesne, M.; Jolles, B.; Sureau, F.; Turpin, P. Y.; Vigny, P. Interaction of Iron-Anthracycline Complexes with Living Cells: a Microspectrofluorometric Study. *Biochem. Biophys. Acta* **1993**, *1177*, 236−244.

(16) Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem. J.* **1953**, *55*, 416−421.

(17) Wang, A. H. J.; Gao, Y. G.; Liaw, Y. C.; Li, Y. k. Formaldehyde cross-links daunorubicin and DNA efficiently: HPLC and X-ray driffraction studies. *Biochemistry* **1991**, *30*, 3812−3815.

(18) Gao, Y. G.; Liaw, Y. C.; Li, Y. k.; van der Marel, G.; van Boom, J. H.; Wang, A. H. J. Facile formation of a crosslinked adduct between DNA and the daunorubicin derivative MAR70 mediated by formaldehyde: molecular structure of the MAR70-d(CGTnACG) covalent adduct. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 4845−4849.

(19) Zhang, H.; Gao, Y. G.; van der Marel, G. A.; van Boom, J. H.; Wang, A. H. Simultaneous incorporations of two anticancer drugs into DNA. The structures of formaldehyde-cross-linked adducts of daunorubicin-d(CG(araC)GCG) and doxorubicin-d(CA(araC)GTG) complexes at high resolution. *J. Biol. Chem.* **1993**, *268*, 10095−10101.

(20) Shiraishi, H.; Kataoka, M.; Morta, Y.; Umemoto, J. Interaction of hydroxyl radicals with tris(hydroxymethyl)aminomethane and Good's buffers containing hydroxylmethyl or hydroxylethyl residues produce formaldehyde. *Free Rad. Res. Commun.* **1993**, *19*, 315.

(21) Cullinane, C.; Cutts, S. M.; van Rosmalen, A.; Phillips, D. R. Formation of adriamycin--DNA adducts in vitro. *Nucleic Acids Res.* **1994**, *22*, 2296−2303.

(22) Arcamone, F. *Doxorubicin Anticancer Antibiotics*; Academic Press: New York, 1981; pp 246−254.

(23) Skladanowski, A.; Konopa, J. Relevance of interstrand DNA crosslinking induced by anthracyclines for their biological activity. *Biochem. Pharmacol.* **1994**, *47*, 2279−2287.

(24) Skladanowski, A.; Konopa, J. Interstrand DNA crosslinking induced by anthracyclines in tumour cells. *Biochem. Pharmacol.* **1994**, *47*, 2269−2278.

(25) Myers, C. E.; Gianni, L.; Simone, C. B.; Klecker, R.; Greene, R. Oxidative destruction of erythrocyte ghost membranes catalyzed by the doxorubicin-iron complex. *Biochemistry* **1982**, *21*, 1707−1712.

(26) Myers, C.; Gianni, L.; Zweier, J.; Muindi, J.; Sinha, B. K.; Eliot, H. Role of iron in adriamycin biochemistry. *Fed. Proc.* **1986**, *45*, 2792−2797.

(27) Leng, F.; Savkur, R.; Fokt, I.; Przewloka, T.; Priebe, W.; Chaires, J. B. Base specific and regiospecific chemical cross-linking of Daunorubicin to DNA. *J. Am. Chem. Soc.* **1996**, *118*, 4731−4738.

(28) Thorndike, J.; Beck, W. S. Production of formaldehyde from N5-methyltetrahydrofolate by normal and leukemic leukocytes. *Cancer Res.* **1977**, *37*, 1125−1132.

(29) Ebeler, S. E.; Zulim, R. A.; Hinrichs, S. H.; Clifford, A. J.; Shibamoto, T. Tumor-bearing transgenic mice and cancer patients have elevated levels of expired formaldehyde. *Faseb J.* **1993**, *7*, A716.

(30) Bagchi, D.; Bagchi, M.; Hassoun, E. A.; Kelly, J.; Stohs, S. J. Adriamycin-induced hepatic and myocardial lipid peroxidation and DNA damage, and enhanced excretion of urinary lipid metabolites in rats. *Toxicology* **1995**, *95*, 1−9.

(31) Bucciarelli, E. Mammary tumor induction in male and female Sprague-Dawley rats by Adriamycin and daunomycin. *J. Natl. Cancer Inst.* **1981**, *66*, 81−84.

(32) Purewal, M.; Liehr, J. G. Covalent modification of DNA by daunorubicin. *Cancer Chemother. Pharmacol.* **1993**, *33*, 239−244.

(33) Bartoszek, A.; Wolf, C. R. Enhancement of doxorubicin toxicity following activation by NADPH cytochromeP450 reductase. *Biochem. Pharmacol.* **1992**, *43*, 1449−1457.

(34) Averbuch, S. D.; Gaudiano, G.; Koch, T. H.; Bachur, N. R. Radical dimer rescue of toxicity and improved therapeutic index of Adriamycin in tumor-bearing mice. *Cancer Res.* **1985**, *45*, 6200−6204.

(35) Acton, E. M.; Tong, G. L.; Mosher, C. W.; Wolgemuth, R. L. Intensely Potent Morpholinyl Anthracyclines. *J. Med. Chem.* **1984**, *27*, 638−645.

(36) Uchida, T.; Imoto, M.; Takahashi, Y.; Odagawa, A.; Sawa, T.; Tatsuta, K.; Naganawa, H.; Hamada, M.; Takeuchi, T.; Umezawa, H. New potent anthracyclines, barminomycins I and II. *J. Antibiot.* **1988**, *41*, 404−408.

(37) Ikeda, D.; Ajito, K.; Kondo, S.; Takeuchi, T. Synthesis of 3″-dehydro-4-O-methylbarminomycin II having an eight-membered acetal-azomethine ring. *Chem. Lett.* **1990**, 1431−1432.

(38) Nagy, A.; Armatis, P.; Schally, A. V. High yield conversion of doxorubicin to 2-pyrrolinodoxorubicin, an analog 500−1000 times more potent: Structure-activity relationship of daunosamine-modified derivatives of doxorubicin. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 2464−2469.

(39) Zwelling, L. A.; Altschuler, E.; Cherif, A.; Farquhar, D. N-(5,5-Diacetoxypentyl)doxorubicin: A novel anthracycline producing DNA interstrand cross-linking and rapid endonucleolytic cleavage in human leukemia cells. *Cancer Res.* **1991**, *51*, 6704−6707.

(40) Cherif, A.; Farquhar, D. N-(5,5-Diacetoxypent-1-yl)doxorubicin: A New Intensely Potent Doxorubicin Analogue. *J. Med. Chem.* **1992**, *35*, 3208−3214.

(41) Walker, J. F. *Formaldehyde*, 3rd ed.; Reinhold: New York, 1964; pp 244−245.

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