Sequence Specificity of Adriamycin-DNA Adducts in Human Tumor Cells[1]

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Abstract

The anticancer anthracycline compound Adriamycin is a known topoisomerase II inhibitor but is also capable of exerting other cellular consequences. After intercalation, Adriamycin can form covalent adducts with DNA, and the magnitude of these adducts appears to be limited by the cellular availability of formaldehyde. Adducts produced by Adriamycin in the presence of formaldehyde have been well characterized in cell-free systems but not in cells. In this study, we show that when Adriamycin is used in conjunction with the formaldehyde-releasing prodrug AN-9 in IMR-32 tumor cells, this allows the formation of sufficiently high levels of adducts in genomic DNA to enable detection of their DNA sequence specificity for the first time. The 340-bp α-satellite *Eco*RI repeat sequence was isolated from drug-treated cells and digested with λ-exonuclease to determine adduct sites at which exonuclease digestion was blocked. The Adriamycin adducts were formed predominantly at 5′-GC and GG sequences and unstable with respect to elevated temperatures and extended times at 37°C. The use of three anthracycline derivatives lacking a 3′amino group demonstrated that this amino portion is critical for the formation of anthracycline adducts in cells. The structure of these drug-DNA adducts can therefore be considered to be identical to the Adriamycin adducts, which have been characterized rigorously in cell-free systems by X-ray crystallography, two-dimensional nuclear magnetic resonance, and mass spectrometry.

Introduction

The anthracycline group of antitumor antibiotics is used widely for cancer chemotherapy, where agents, such as Adriamycin (Fig. 1), daunomycin, epirubicin, and idarubicin, have proved the most useful compounds. The various neoplasms that the anthracyclines are effective against include acute leukemias and solid tumors, such as carcinomas of the breast, lung, thyroid, and ovary, and soft tissue sarcomas (1). The anthracyclines are classified as topoisomerase II inhibitors because they effectively stabilize the cleavage complex of topoisomerase II at clinical concentrations (2). However, their cellular effects are by no means limited to this type of enzymatic inhibition. Although it is clear that their antitumor activity is consistent with their ability to interact with cellular DNA, this noncovalent interaction can inhibit the function of DNA ligases, helicases, and other DNA-dependent proteins, in addition to topoisomerase II (2). Another important mechanism may be redox cycling of the quinone portion of the drug, leading to the production of radical species, which can directly damage DNA or cell membranes, causing DNA strand scission or lipid peroxidation (3). Indeed, it is this radical generating mechanism that is a major cause of the cardiotoxicity that is a serious side effect of high dosages of Adriamycin.

Another type of DNA interaction involves activation of the drug by cellular formaldehyde, leading to the formation of covalent DNA adducts. The structure of these adducts in cell-free systems has been established independently by a number of research groups and involves the intercalated drug becoming fixed to the DNA via a formaldehyde-derived bridge, such that the amine group of the daunosamine sugar of Adriamycin is covalently attached to the N-2 of guanine (4–6). These adducts form predominantly at 5′-GC sequences and stabilize the structure of dsDNA,[3] although they do not form classical interstrand cross-links (7). In early studies which examined the capacity of Adriamycin to form DNA adducts, and which indeed demonstrated low-level drug reactivity *in vitro,* the alternative reductive activation systems and proposed mechanisms of reaction implied a different structure to the formaldehyde-mediated adducts. The alkylating intermediates in these studies were proposed to be either aglycone quinone-methide or C-7-centered radical species (8, 9), clearly different to the formaldehyde-activated intermediate that retains the daunosamine sugar. The chemical nature of these alternative adducts has proved elusive, although studies with the anthracycline derivative menogaril have shown that the reductively activated quinone methide form binds covalently to the N-2 of deoxyguanine (10).

[3] The abbreviations used are: dsDNA, double-stranded DNA; Tm, melting temperature.
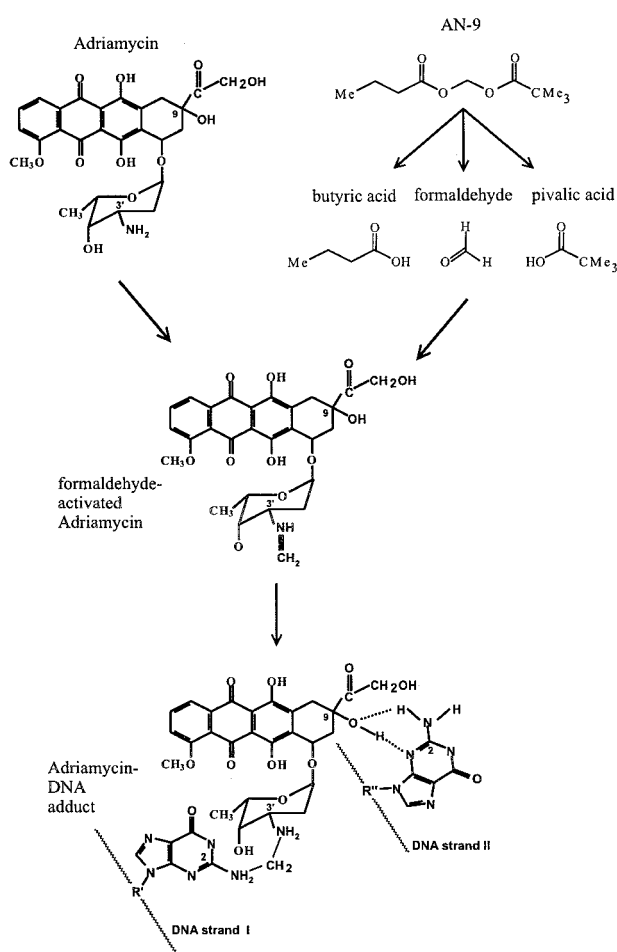
Fig. 1. Structures of Adriamycin and AN-9 and pathway of activation of Adriamycin by formaldehyde released by esterase-mediated hydrolysis of AN-9.

Although formaldehyde-mediated Adriamycin adducts can form easily in artificial systems, where concentration and purity of components required for the reaction are optimal, proving their existence at relevant concentrations in intact cells has been more difficult. A series of studies has shown that Adriamycin can stabilize the structure of dsDNA in tumor cells and also [^{14}C]-Adriamycin can react covalently with cellular DNA (11, 12). Furthermore, the observation that Adriamycin catalyzes the formation of formaldehyde in cells provides an insight into how the adducts may occur in cellular DNA (13). However, it is still unclear if the types of structures recognized in cells actually reflect a formaldehyde-mediated structure or an alternative structure. The formaldehyde-conjugate of Adriamycin, doxoform, has been shown to be more cytotoxic than the parent compound, and DNA-covalent binding has been implicated as its mechanism of action, although these species have not been characterized in cellular DNA (14).

Recently, we have shown that Adriamycin with the butyric acid-releasing prodrug AN-9 is an effective combination to produce high levels of Adriamycin adducts in tumor cells

(15). The synergy displayed by this combination represents a promising therapeutic alternative to the use of Adriamycin alone, and indeed, proof of principle has been obtained in a mouse leukemia model (16). We have demonstrated that enhanced Adriamycin adduct formation facilitated by the combination is mainly caused by the formaldehyde, which is also released by AN-9, rather than the released butyric acid (Fig. 1). Thus, it appears that the cellular availability of formaldehyde represents a rate-limiting process for intracellular adduct formation. The use of Adriamycin in conjunction with the formaldehyde-releasing prodrug has allowed the formation of sufficiently high levels of adducts in cells to demonstrate their DNA sequence specificity for the first time. We show here that these cellular adducts have the same characteristics as those observed previously in cell-free systems and conclude that Adriamycin-DNA adducts detected by formaldehyde activation in cells are the same as those which have been well characterized *in vitro* by X-ray diffraction, nuclear magnetic resonance, and mass spectrometry.

Materials and Methods

Materials. Adriamycin hydrochloride and anthracycline derivatives were a gift from Farmitalia Carlo Erba (Milan, Italy). Pivaloyloxymethyl butyrate (AN-9) was synthesized as described previously (17). Porcine liver carboxyl esterase was obtained from Sigma-Aldrich. Adriamycin stocks were dissolved in H$_2$O, whereas other drugs were dissolved in methanol.

Cell Culture and Drug Treatment of Cells. IMR-32 cells were maintained in DMEM containing 10% FCS. Cells were maintained at 37°C in 5% CO$_2$. On the day before experiments, cells were seeded at a density of 1.5×10^6 cells/10-cm Petri dish and allowed to attach overnight to ensure that cells were in exponential growth phase at the time of drug treatment. Cells were treated with 4 μM Adriamycin and 500 μM AN-9 for a total of 4 h (the AN-9 was added 2 h after Adriamycin), then harvested and washed twice with PBS. Total genomic DNA was extracted using a QIAamp blood kit (Qiagen) where the lysis procedure was carried out at 50°C for 30 min to minimize loss of adducts.

Isolation of 296-bp α-Satellite DNA Fragment. Genomic DNA was restriction digested with *Eco*RI and resolved electrophoretically using a 6% nondenaturing polyacrylamide gel. Electrophoretic migration of the 340-bp α-satellite repeat was determined by ethidium bromide staining, and this fragment was excised. DNA was eluted from gel slices by overnight incubation in 0.5 M ammonium acetate and 1 mM EDTA at 4°C. Ethidium bromide was removed by phenol/chloroform extraction, and then samples were ethanol precipitated and resuspended in Tris-EDTA buffer. DNA fragments were 3′ end labeled with [^{32}P]-dATP using the Klenow fragment of DNA polymerase and then restriction digested using *Hae*III. The 296-bp band was separated using a 5% nondenaturing polyacrylamide gel and located by conducting a short exposure of the gel to Kodak X-OMAT AR-5 film and then excised and eluted overnight in ammonium acetate buffer. Samples were exposed to phenol/chloroform extraction, ethanol precipitated, and resuspended in Tris-
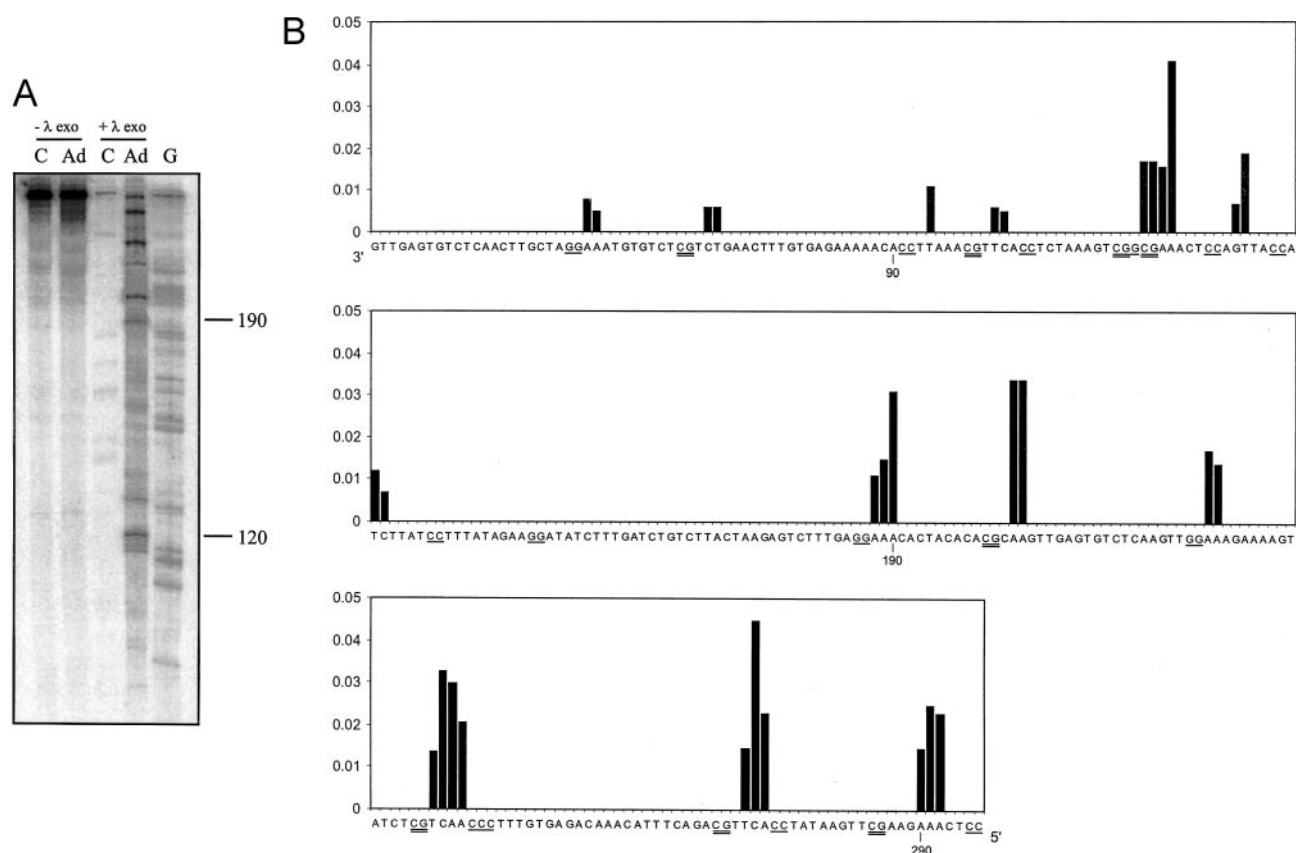
Fig. 2.   DNA sequence specificity of Adriamycin and AN-9 in α DNA fragments. In *A*, the 296-bp fragment was isolated from cells treated with Adriamycin and AN-9 (*Ad*) and cells treated with 500 μM AN-9 only (*C*) and was subsequently end labeled. Samples were untreated (−λ exo) or digested with λ exonuclease (+λ exo) and resolved using a 10% denaturing polyacrylamide gel. A Maxam-Gilbert G sequencing lane is also shown (*G*). In *B*, the *lane* corresponding to the Adriamycin-AN-9 (*Ad*) sample treated with exonuclease was quantitated by PhophorImage analysis to determine the relative intensity of each band corresponding to a drug blockage site. Each blockage site is represented as the mol fraction of total blockages at the corresponding sequence. The sequence is written as 3′-5′, and 3′-CG (5′-GC) sites are shown with double underlining, whereas 3′-CC (5′-GG) and 3′-GG (5′-CC) sites are shown with single underlining. Identical blockage sites for the drug combination were observed in four independent experiments.

EDTA buffer. The radiolabelled 296-bp fragments were digested at 37°C for 1.5 h using λ-exonuclease to progressively release 5′ nucleotides. Maxam-Gilbert G sequencing was conducted as described previously (18). Samples were resolved by denaturing electrophoresis through 10% sequencing gels. Samples were also subjected to periods of extended electrophoresis for better sequence resolution.
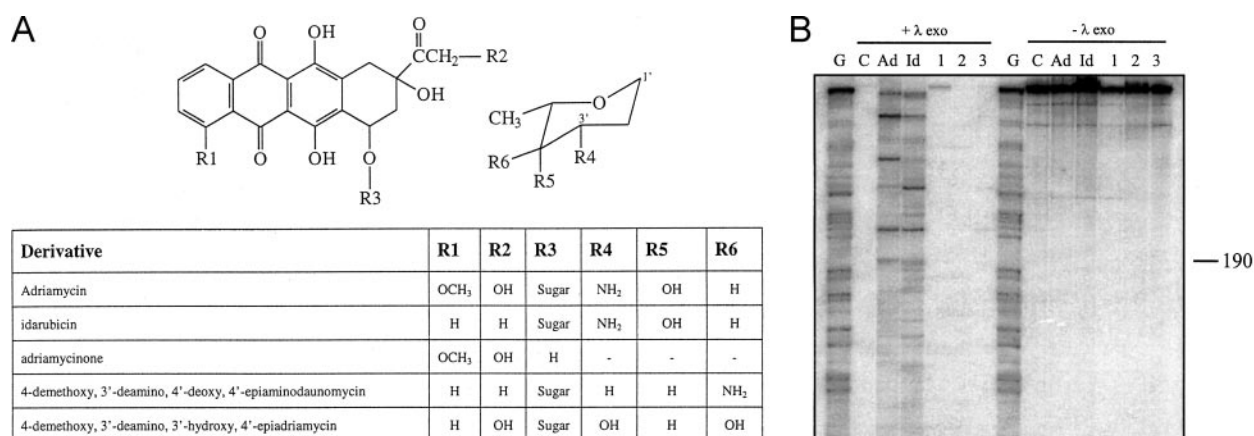
Treatment of Cells with Radiolabelled Drug. IMR-32 cells were seeded at a density of 2.5×10^6 cells/10-cm Petri dish. The following day, cells were pretreated with 4 μM [^{14}C]-Adriamycin for 2 h and then treated with 260 μM AN-9 for an additional 2 h. Cells were then harvested, and genomic DNA was isolated using a QIAamp blood kit. Residual intercalated Adriamycin was removed by two phenol extractions, one chloroform extraction and ethanol precipitation. Samples were exposed to various times of standing at 37°C or various temperatures for 10 min, and unstable adducts represented by unbound drug were removed by an additional phenol/chloroform extraction. Residual [^{14}C]-Adriamycin represented remaining adducts and was assessed by scintillation counting.

Esterase Activation of AN-9 in a Cell-free System. Genomic DNA was extracted from untreated IMR-32 cells using the QIAamp procedure. This DNA (50 μM bp) was reacted with 5 μM [^{14}C]-Adriamycin, 5 mM AN-9, and 200 μg/ml esterase for 5 h at 37°C in 1 × Tc buffer [1 × Tc; 40 mM Tris (pH 8.0), 3 mM MgCl$_2$, 100 mM KCl, and 0.1 mM EDTA]. An alternative activation system used 2 mM formaldehyde in place of AN-9 and esterase. Samples were subsequently extracted and processed as outlined above.

Effect of DNA Methylation on Adduct Formation. The plasmid pCDNA1 (Invitrogen, San Diego, CA) was methylated at 5′-CG sequences using S*ss1* methylase. DNA (methylated and unmethylated) was reacted with 2 μM [^{14}C]-Adriamycin and 2 mM formaldehyde in 1 × Tc for times of 0–3 h. Samples were phenol/chloroform extracted, ethanol precipitated, and assessed for incorporation of [^{14}C]-Adriamycin by scintillation counting.

Gene-specific dsDNA Stabilization Assay. IMR-32 cells were treated with Adriamycin and AN-9 at concentrations that induced ~60–80% dsDNA stabilization of the mitochondrial DNA genome. DNA samples were then exposed to

A

Derivative	R1	R2	R3	R4	R5	R6
Adriamycin	OCH₃	OH	Sugar	NH₂	OH	H
idarubicin	H	H	Sugar	NH₂	OH	H
adriamycinone	OCH₃	OH	H	-	-	-
4-demethoxy, 3'-deamino, 4'-deoxy, 4'-epiaminodaunomycin	H	H	Sugar	H	H	NH₂
4-demethoxy, 3'-deamino, 3'-hydroxy, 4'-epiadriamycin	H	OH	Sugar	OH	H	OH

B

Fig. 3.   Ability of sugar-modified anthracycline derivatives to block λ exonuclease. *A,* structure of idarubicin and sugar-modified anthracyclines. In *B,* the 296-bp DNA fragment was isolated from cells treated with a combination of 500 μM AN-9 and either 4 μM Adriamycin (*Ad*), idarubicin (*Id*), 4-demethoxy 3'-deamino 4'-deoxy 4'-epi amino daunorubicin (*1*), 4-demethoxy 3'-deamino 3'-hydroxy 4'-epi doxorubicin (*2*), or Adriamycinone (*3*). Control samples (*C*) that were untreated are also shown. Samples were either analyzed directly (−λ exo) or exposed to λ exonuclease digestion (+λ exo). The same exonuclease blockage sites for all drugs were observed in two independent experiments.

various times of standing at 37°C or various temperatures for 10 min. Samples were processed for Southern analysis of the mitochondrial genome as described previously (15).
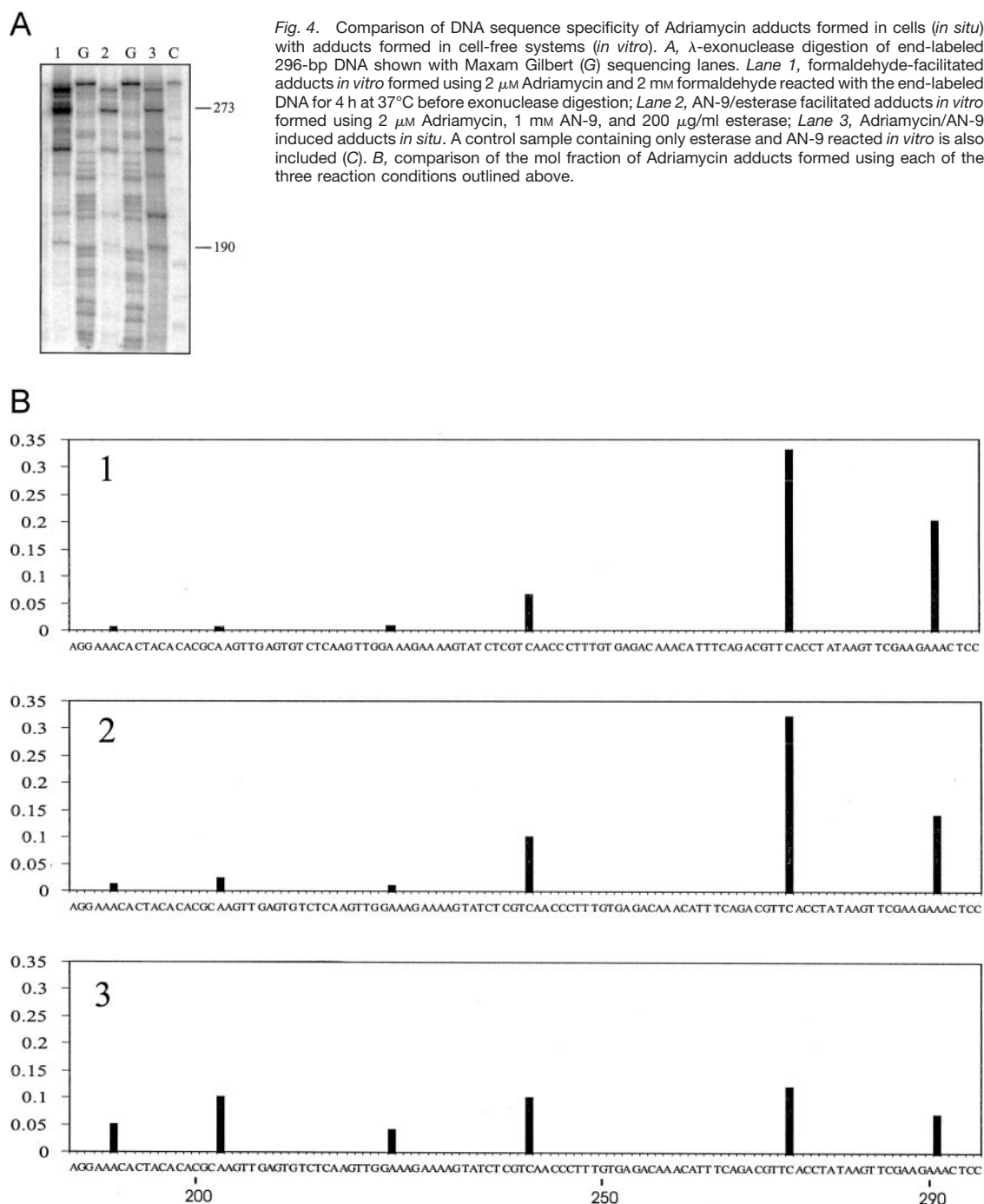
Results

Adriamycin Adducts in Cells Are GC Specific. To assess the DNA sequence specificity of Adriamycin, the 296-bp α-satellite fragment was isolated from IMR-32 cells treated with Adriamycin and AN-9 and then subjected to λ-exonuclease digestion. Adducts cause direct blockages to the stepwise digestion of λ-exonuclease, and the remaining lengths of radiolabelled fragment therefore reveal the location of adduct-binding sites. The digestion patterns of λ- exonuclease are shown in Fig. 2*A,* where the only visible truncated digestion products are in the lane treated with both Adriamycin and AN-9. The control lane (AN-9 only) produced no visible blockages (nor did cells treated with Adriamycin in the absence of AN-9; data not shown). Samples which were not treated with exonuclease showed that the drug combination did not produce any single-strand nicks in the DNA. The sequence specificity of blockages produced by the Adriamycin/AN-9 combination in parallel with Maxam-Gilbert G sequencing is shown in Fig. 2*B.* The nonambiguous sequencing lane obtained confirms the absence of significant contaminating DNA sequences. The striking 5'-GC specificity of the combination is very similar to that observed previously in experiments with Adriamycin in cell-free systems (7, 19, 20). It is apparent that the adducts occur before every 5'-GC sequence examined. Of the 13 groups of blockages represented in Fig. 2*B,* this includes all eight of the 5'-GC

(double underlining) sites represented and 5 of the 12 5'-CC or 5'-GG sites (single underlining) within the fragment that were analyzed. However, at one site, it is not possible to tell which sequence contributes most to the blockage in exonuclease digestion (5'-GCGGC). The blockage to exonuclease digestion generally occurs 2–4 bp before the site of adduct formation. To ensure that adducts obtained represent reaction processes which have occurred within viable cells, a small proportion of cells at the end of each treatment period was assessed for viability. In control cells where no drug was used for treatment, viability as assessed by trypan blue exclusion was 99%, whereas cells treated with 4 μM Adriamycin and 500 μM AN-9 also exhibited a high proportion of viable cells (88%). Furthermore, DNA extracted from all groups of treated cells showed no evidence of apoptotic laddering.

Anthracycline Derivatives Confirm Necessity of 3'Amino for Observed Sequence Specificity. Cells were treated with a combination of AN-9 and various anthracycline derivatives (Fig. 3*A*) and then analyzed for sequence specificity within the 296-bp fragment (Fig. 3*B*). This demonstrates that idarubicin possesses the same sequence specificity as Adriamycin, whereas two 3'deamino derivatives and the Adriamycin aglycone (adriamycinone) do not yield any discernable specificity.

Adducts Exhibit the Same Specificity in Cells when Compared with Cell-free Systems. Fig. 4 shows a comparison of truncated exonuclease fragments produced in cells with those obtained in cell-free systems. Adducts were induced in a cell-free system by activating Adriamycin in the presence of formaldehyde and DNA, and an alternative ac-

Fig. 4. Comparison of DNA sequence specificity of Adriamycin adducts formed in cells (*in situ*) with adducts formed in cell-free systems (*in vitro*). *A*, λ-exonuclease digestion of end-labeled 296-bp DNA shown with Maxam Gilbert (*G*) sequencing lanes. *Lane 1,* formaldehyde-facilitated adducts *in vitro* formed using 2 μM Adriamycin and 2 mM formaldehyde reacted with the end-labeled DNA for 4 h at 37°C before exonuclease digestion; *Lane 2,* AN-9/esterase facilitated adducts *in vitro* formed using 2 μM Adriamycin, 1 mM AN-9, and 200 μg/ml esterase; *Lane 3,* Adriamycin/AN-9 induced adducts *in situ*. A control sample containing only esterase and AN-9 reacted *in vitro* is also included (*C*). *B,* comparison of the mol fraction of Adriamycin adducts formed using each of the three reaction conditions outlined above.

tivation procedure was also used to simulate the intracellular release of formaldehyde by AN-9. Because AN-9 is cleaved by cellular esterases to produce one molecule each of butyric acid, formaldehyde, and pivalic acid (17), the alternative procedure involved incubating Adriamycin in the presence of AN-9, esterase, and DNA. The exonuclease blockage proportions produced by Adriamycin and formaldehyde are the same as that produced by Adriamycin, esterase, and AN-9. This is consistent with the fact that it is the formaldehyde released from AN-9 that activates Adriamycin to form ad-

ducts. When compared with the blockage profile of Adriamycin/AN-9-induced adducts in cells, it is apparent that sites of blockages are exactly the same as detected in the cell-free systems with the most pronounced blockage sites being 5′-GC and 5′-GG sequences (Fig. 4B), and this is consistent with the specificities observed in previous publications. However, the intensity of the blockages is different in cells with the GG sequences displaying a higher adduct occupancy and generally a more even distribution of adduct sites. This is probably caused by high levels of adduct formation in
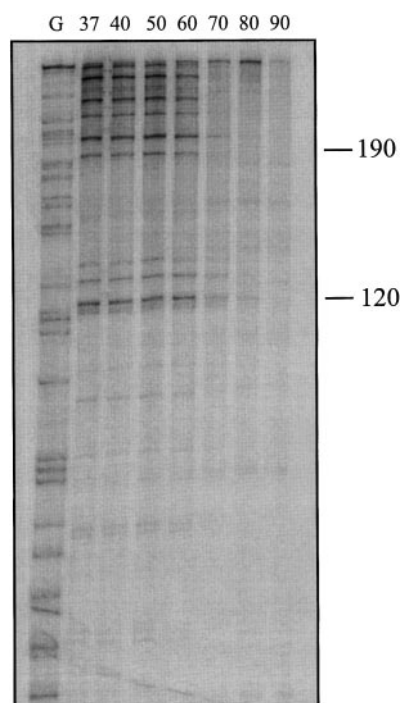
Fig. 5. Thermal denaturation of site-specific Adriamycin adducts. The radiolabeled 296-bp fragment was isolated from cells treated with Adriamycin and AN-9. Samples were subjected to 10-min incubations at temperatures ranging from 37°C to 90°C before being digested with λ-exonuclease and resolved using a 10% gel. The same temperature-dependent digestion profile was obtained in two independent experiments.

Table 1 Thermal denaturation profile of Adriamycin adducts in relation to sequence specificity

PhosphorImage analysis of the gel in Fig. 5 enabled determination of Tms of individual blockage sites. The decreasing intensity of the band corresponding to each blockage site was plotted, and the temperature at which 50% of the intensity of the band remained was determined as the Tm. The 5-bp sequence shown is the sequence located 1 nucleotide before each group of blockages presented in Fig. 2B.

Site	Fragment length	Sequence	Tm
1	120	CGGCG	68.8 ± 1.4
2	128	CTCCA	69.1 ± 1.7
3	134	TACCA	68.7 ± 3.6
4	190	TGAGG	65.3 ± 2.4
5	203	CACGC	63.7 ± 2.4
6	224	GTTGG	63.6 ± 2.4
7	241	TCTCG	61.6 ± 1.7
8	273	GACGT	59.5 ± 1.7
9	291	CGAAG	60.8 ± 1.5

the cell-free experiments rather than reflecting a difference in preference for adduct sites in cells.

CG Methylation Does Not Influence Adriamycin Adduct Formation. To test whether altered DNA structure, such as cytosine-methylated 5′-CG DNA sequences, may play a role in Adriamycin adduct formation in cells, CG sites of plasmid DNA were methylated. Adducts produced by the combination of Adriamycin and formaldehyde were compared with unmethylated DNA, and there was no difference in adduct
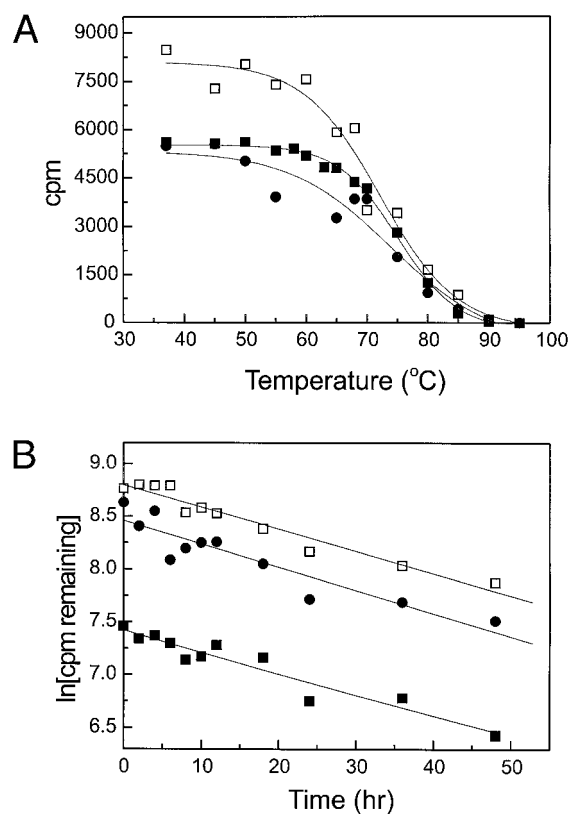

Fig. 6. Stability of [^{14}C]-Adriamycin adducts. *A,* thermal lability of adducts formed with genomic DNA. The condition for formation of adducts was formaldehyde-facilitated adducts *in vitro* (□), AN-9/esterase-facilitated adducts *in vitro* (●), and Adriamycin/AN-9-induced adducts *in situ* (■). In *B,* first order decay of Adriamycin adducts formed under the same conditions.

formation using either of the DNA sources at any of the time points examined. The amount of adducts at the completion of a 3-h reaction (duplicate samples) were 22.2 ± 0.4 adducts/10 kb with unmethylated DNA, compared with 21.1 ± 0.1 adducts/10 kb with methylated DNA. Samples taken at intervals before the 3-h completion also revealed an identical rate of reaction.

Sequence-specific Adriamycin Adducts in Cells Are Temperature Labile. To further characterize the Adriamycin adducts formed in cells, the 296-bp fragment was isolated from cells treated with Adriamycin and AN-9 and exposed to various temperatures before exonuclease digestion (Fig. 5). This enabled the Tm for loss of adducts at individual sites to be determined (Table 1). These temperatures ranged from 60°C to 70°C, similar to those observed previously for Adriamycin adducts in a cell-free environment. This confirms that the sequence-specific Adriamycin adducts formed in cells are most likely of a similar structure to those formaldehyde-mediated adducts, which have been characterized previously in cell-free systems (4, 20). The same melting profiles were obtained in a separate biological experiment for both Adriamycin and the related compound idarubicin. Although the results for site-specific adduct populations revealed a similar lability to

Table 2 Summary adduct Tm and half-life (37°C) determinations

Tm and half-life values for Adriamycin adducts were determined using data from Figs. 6 and 7. Melting profiles and half-lives were estimated by least squares fitting to sigmoidal and linear decays, respectively, and the SE of fit is shown.

| Treatment	Tm (^{14}C)	Tm (Southern)	T½ (^{14}C)	T½ (Southern)
Adr/formaldehyde (cell free)	72.2°C ± 1.6	72.1°C ± 2.3	33.2 h ± 2.7	31.5 ± 1.8
Adr/AN-9 (cell free)	74.3°C ± 3.5	75.6°C ± 4.0	31.7 h ± 4.4	28.1 ± 1.7
Adr/AN-9 (cells)	75.1°C ± 0.4	73.5°C ± 7.7	34.0 h ± 3.0	29.1 ± 2.9

that reported previously, these are all lower than the Tm for the release of the chromophore from bulk DNA (Fig. 6). This phenomenon has also been reported previously (20). The effect of flanking sequences on the Tm of each sequence cannot be elucidated because of the apparent overriding effect of where the adduct site is in relation to the length of the fragment (*i.e.,* the adducts encountered first by the exonuclease display a lower Tm than those recognized last).

Adducts Isolated from Cells Exhibit the Same Tm and Half-Life at 37°C As Adducts Formed in Cell-free Systems. To confirm that the DNA adducts induced in cells by AN-9 were the same as those produced in cell-free experiments, [^{14}C]-Adriamycin was used to form adducts under three different conditions: (*a*) adducts formed in cells and adducts formed in cell-free systems using either (*b*) AN-9 and esterase or (*c*) formaldehyde to activate Adriamycin. This allowed the fate of the Adriamycin chromophore in response to elevated temperature and extended times at 37°C to be assessed. The results of this study are presented in Fig. 6 and summarized in Table 2. Adducts were purified from the three different environments, and all showed similar thermal lability (Fig. 6*A*) and first order decay kinetics (Fig. 6*B*), confirming that the adducts in cells are most likely of identical structure to those produced *in vitro*.

The adducts were also measured as virtual cross-links (*i.e.,* their ability to stabilize dsDNA) and displayed similar thermal stability (Fig. 7*B*) and first order decay kinetics (Fig. 7*C*) as detected by a mitochondrial DNA-specific Southern hybridization assay.

Discussion

For any agent that damages DNA, it is relevant to study its interactions with purified naked DNA. However, ultimately, it is important to characterize the DNA interaction in a cellular environment where the DNA may be less accessible because of compartmentalization and complex interactions with nucleosomes and other chromatin-associated proteins. In addition, the drug may produce alternative metabolic intermediates in cells that may produce a different sequence specificity. For the purpose of analyzing genomic specificity, the most routinely studied DNA sequence in human cells is the high-copy 340-bp tandem repeat of α-satellite DNA, which constitutes ~0.75% of the genome (21). This transcriptionally inert sequence is predominantly located at the centromeres of human chromosomes. Overall, despite differences in DNA replication timing, DNA damage repair, DNA methylation, and histone modification compared with other genomic regions, this sequence represents a good fragment
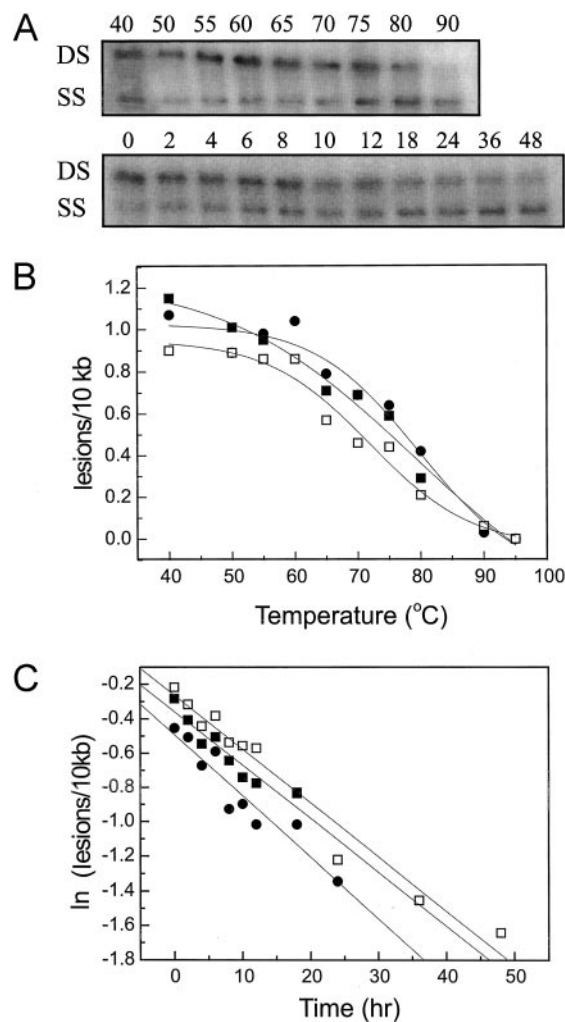
Fig. 7. Stability of Adriamycin adducts using Southern hybridization. *A,* representative Southern blots for stability of adducts with increasing temperature (40°C–90°C) as shown (*top panel*) and time-dependent stability (0–48 h) at 37°C (*bottom panel*). The positions of migration of dsDNA (*DS*) that represents adducts and single-stranded DNA (*SS*) are indicated. *B,* thermal lability of adducts formed with genomic DNA. The conditions for adduct formation were the same as outlined in Fig. 6. *C,* first order decay of Adriamycin adducts.

(because of its abundance) to study the specificity of DNA damage in intact cells. Furthermore, although each individual sequence varies with respect to a consensus sequence with an average of 8.8% divergence and a range of 1–21% (22, 23), the homogeneity is such that the cellular DNA sequence

specificity of many drugs has been elucidated using this repeat sequence (24).

The α-satellite repeat sequence has been used to study the cellular DNA specificity of bleomycin-induced, single-stranded breaks, nitrogen mustard-induced piperidine cleavage, and Taq polymerase-based detection of platinum coordination complexes (24). However, none of these techniques are suitable for the detection of damage induced by Adriamycin because anthracycline adducts cannot be induced to produce strand breaks after covalent binding and are not amenable to PCR-based detection techniques because of their heat lability. It was found that a suitable technique to detect cyanomorpholinoadriamycin adducts was isolation of the 340-bp repeat followed by exonuclease III digestion (25). This exonuclease, along with λ-exonuclease, are suitable enzymes to detect damage induced by Adriamycin adducts. However, in the same study, Adriamycin adducts could not be detected in cells.

In the current study, the 296-bp sequence was isolated under conditions which subjected DNA to low temperature (4°C) so as to minimize loss of adducts. There is reportedly no loss of adducts under these conditions (6). Even so, it has not been possible to detect adducts induced by Adriamycin alone. However, the inclusion of AN-9 has allowed the production of sufficient levels of adducts to enable their DNA sequence specificity in cells to be determined for the first time. This specificity is similar to that observed previously in cell-free experiments in that 5′-GC and GG sites are the most pronounced blockage sites. However, the high-intensity GG blockages have not been observed previously. This may represent a saturation of drug-binding sites because of the high concentrations of Adriamycin and AN-9 used in this work or a greater stability of adducts at these sites. These observations offer a possibility for telomeric DNA (TTAGGG)$_n$ and G quadruplexes (26) as potential hot spots for damage by Adriamycin adducts. Adducts at 5′-GC sites have been extensively characterized structurally (4–6). The structure of the adduct will accommodate a GG sequence because covalent binding to the N-2 of guanine on only one strand is required, whereas the other strand accommodates H-bonding (6). The observation that anthracyclines lacking the amino sugar or amino portion of the sugar do not form sequence-specific adducts is fully consistent with the known structure of the adduct. It should be noted that the inherent capacity of a drug to form such adducts does not correlate with their cytotoxicity as single agents (i.e., in the absence of formaldehyde-releasing prodrugs) because the aglycone is relatively inactive, and the 3′deamino derivatives have enhanced activity in situ (27).

In a previous study, enhancement of Adriamycin adduct formation by AN-9 was shown to be absolutely dependent on formaldehyde release by the prodrug (15). The use of a series of prodrug derivatives showed that the release of formaldehyde was required for enhanced adduct formation, whereas acetaldehyde release abolished this enhancement. Semicarbazide sequestration of formaldehyde in these experiments abrogated adduct formation and reversed cytotoxicity, thus confirming the direct role for formaldehyde-mediated adduct formation in the cytotoxic response mediated by Adriamycin and AN-9. The use of AN-9 with esterase in the cell-free system in the current study represents a model system for the esterase-mediated cleavage of AN-9 in cells. Our study has shown that the adducts formed in cells have the same characteristics as have been observed previously in cell-free systems using formaldehyde (4, 20) and in the AN-9/esterase cell-free system. It is unclear whether the background levels of Adriamycin adducts in cells obtained in the absence of prodrug (not detectable by the assay used in the current study) represent formaldehyde-mediated adducts or an alternative structure that has been hypothesized previously (8, 9, 11). It is likely that they are formaldehyde-mediated because endogenous formaldehyde is present in tumor cells, and cellular formaldehyde production is stimulated by Adriamycin itself (13).

It is relevant to consider the structure of chromatin relative to naked DNA, because this may influence the site specificity of Adriamycin adducts. It appears that the specificity of Adriamycin adducts in purified versus cellular DNA is similar, and this observation is consistent with many other DNA-damaging agents (24). Some studies have revealed a small preference of DNA-damaging agents for linker compared with nucleosomal regions (28); however, a comparison in the current study is not possible because of the short sequences used and the fact that it is unclear whether nucleosomes in such repetitive DNA are phased with respect to DNA sequences (24, 28–30). A precisely phased nucleosome would be required for such a study. Histones and other DNA-packaging proteins which contribute to the higher order structure of DNA may have influenced the overall accessibility of Adriamycin to DNA because a larger concentration was required compared with cell-free systems. Studies of Adriamycin intercalation using purified nucleosomes have indeed shown a lowered affinity compared with free DNA (31). Conversely, histones do not appear to provide a good level of protection from bulky carcinogens, such as benzo[a]pyrene diol epoxide (32). Topoisomerase II is unlikely to have influenced the specificity of adducts (short of sequestering available drug), because there is no evidence that sites of topoisomerase II-mediated damage (33) are related to sites of DNA adduct formation.

The methylation status of the α-satellite DNA could possibly have contributed to DNA adduct formation because there is a high frequency of 5′-CG sequences, and these sites are often methylated at the C residue in heterochromatin (34). Although Adriamycin targets 5′-GC and not 5′-CG sequences, it is possible that methylated sequences may have influenced adduct formation through indirect means or when such a site was directly adjacent to an adduct site. Therefore, the occurrence of adducts in methylated DNA versus nonmethylated DNA was determined experimentally. Methylation was not found to contribute to adduct formation. This is in contrast to drugs, such as mitoxantrone and Mitomycin C, that are specific for 5′-CG sequences and, in particular, methylated 5′-CG sequences (35, 36). This is relevant because it is likely that prodrugs, such as AN-9 (which release formaldehyde and the histone deacetylase inhibitor

butyric acid), themselves modify the methylation status of DNA.

Drug occupancy levels detected in cells using [^{14}C]-Adriamycin at the concentrations of Adriamycin and AN-9 used in this study are ~100 adducts/10 kb (compared with approximately two adducts/10 kb for Adriamycin alone). It should be noted that the amounts of drug used in this study are considered to be at the extreme of clinically relevant concentrations where plasma Adriamycin concentrations exceed 1 μM (37). Although relatively high concentrations of drugs were used to determine adduct sequence specificity (to ensure a high degree of drug occupancy at each relevant DNA site and also account for the inevitable loss of Adriamycin adducts because of their instability), it is likely that much lower concentrations of drugs would produce a sufficient level of adducts to trigger the cell death machinery. Modifications of this assay can now be used to study the genomic persistence of Adriamycin adducts at multiple individual sequences (transcribed and nontranscribed sequences) and also compare their occurrence in Adriamycin-sensitive and -resistant cells. This will likely involve the adaptation of existing assays using ligation-mediated PCR (24). The cellular consequences of these adducts remain to be elucidated. Determining how (and if indeed) adducts are detected and become substrates of the various DNA repair machineries of the cell, and how these adducts may be linked by cellular pathways to the execution of apoptosis, will be a challenging area of future study.

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