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Formation of adriamycin – DNA adducts *in vitro*

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ABSTRACT

Adriamycin is known to induce the formation of adducts with DNA when reacted under *in vitro* transcription conditions. The factors affecting the extent of adduct formation were examined in order to establish the critical components and optimal conditions required for the reaction, and to gain insight into the nature of the DNA–adduct complex. There was a strong dependence on reaction temperature (with a 40-fold increase of adducts at 40–50°C compared to 10°C), pH (maximum adducts at pH 7), but little dependence on the oxygen level. There was an absolute requirement for a reducing agent, with adducts detected with DTT, β-mercaptoethanol and glutathione, maximal adducts were formed at high levels of DTT (5–10 mM). Adducts were also formed with a xanthine oxidase/NADH reducing system, with increasing amounts of adducts detected with increasing NADH; no adducts were detected in the absence of either the enzyme or NADH. Of fourteen derivatives studied, only four yielded a similar extent of adduct formation as adriamycin; there was no absolute requirement for a carbonyl at C13 or hydroxyl at C14. Adducts were also observed with ssDNA but required a longer reaction time compared to dsDNA. The sequence specificity of adduct formation with ssDNA was examined using a primer-extension assay; almost all adducts were associated with a guanine residue. Overall, the results are consistent with a two-step reaction mechanism involving reductive activation of adriamycin, with the activated species then reacting with the guanine residues of either dsDNA or ssDNA.

INTRODUCTION

The history of the discovery and development of the anthracyclines as active anticancer agents has been clearly outlined in an excellent recent review of these compounds (1). Because of the increased antitumour activity and spectrum of activity found for adriamycin (compared to daunomycin) accompanying the addition of just one hydroxyl group, and the need to overcome the cardiotoxicity of these compounds, there has been an intense international effort to find improved derivatives of adriamycin over the last 25 years. The magnitude of this effort can be gauged from the estimated 2,000 or more derivatives that have been synthesised in that time (1). Although many of these derivatives have exhibited a preclinical antitumour activity similar (or superior) to that of adriamycin, with seven now in clinical use in various countries and many more in current clinical trials, none have resulted in a substantial improvement in the clinic over that of the parent drug, adriamycin (1). The fundamental question that must be asked is why such an intense effort has failed to yield a second generation of significantly improved derivatives. The answer to this question is due largely to the fact that the mechanisms of action remain obscure, and it has therefore not been possible to design derivatives in a logical manner based on a clear understanding of the chemistry involved in the functioning of adriamycin.

The possible modes of action of adriamycin have been summarised in a comprehensive review (2). Three likely modes were considered (impairment of topoisomerase II activity; bioreductive activation of the drug; membrane related effects) and these continue to be regarded as the most likely mechanisms of antitumour action (3). While there is good evidence to support some level of contribution from each of these mechanisms (3), the involvement of topoisomerase IIas a common factor to a range of anticancer agents (4,5) has clearly identified the central role of this enzyme in the mode of action of these cytotoxic drugs. The issue continues to remain unclear however for several reasons: the induction of topoisomerase II mediated DNA damage is rapidly repaired following removal of the cytotoxic agent (4,6) whereas DNA damage is known to increase long after removal of these drugs (7); the process of how reversible damage leads to cell death is not understood, and the subsequent steps which are thought to follow (leading to a permanent lesion) have yet to be identified (4,6); some studies have failed to detect significant DNA strand breakage at drug concentrations equal to that of the IC_{50} value (8); drug-induced cytotoxicity is not associated with topoisomerase II-mediated DNA damage in some cells (9,10).

There has been an increasing body of evidence for the formation of drug-DNA adducts in cells treated with adriamycin (11–14). Because of the possible role of bioreductive activation of adriamycin in the mode of action of this drug, there have been many attempts to isolate and characterise DNA adducts resulting from such a process, both *in vitro* and *in situ*. Anthracycline adducts have been shown to be formed from reduction of the drug *in vitro*, and have been shown to bind covalently to nucleic acid components (15) and to involve the C7 position of the reductively activated quinone methide (16). Enzymatically

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activated adducts have also been reported (17–20). Adriamycin can also be activated non-enzymatically under mild conditions required for the *in vitro* transcription of DNA. Long exposure of adriamycin to DNA under these conditions yields long-lived transcriptional blockages (21) and these have since been shown to be due to the the presence of adducts of adriamycin at GpC sites on the DNA (22). The adducts contain the drug chromophore and appear to be activated by a reducing system involving DTT and Fe(III) ions (23). Given the possibility that such adducts may be related to the mechanism of action of adriamycin it is important to be able to isolate such adducts *in vivo*. Before this can be attempted it is necessary to gain a good understanding of the characteristics of these adducts *in vitro*, especially in view of the difficulty of isolating such adducts in the past.

In order to gain some insight into the composition and nature of these adducts we sought to establish which reaction conditions were critical to their formation. We now present a systematic analysis of the effect of each component of the reaction system on the extent of formation of these adducts, and also examine the role of structural requirements of the drug itself.

MATERIALS AND METHODS

Materials

Adriamycin hydrochloride and all anthracycline derivatives were a gift from Farmitalia Carlo Erba, Milan, Italy. [14-^{14}C]Adriamycin hydrochloride (specific activity, 55 μCi/mmol) was purchased from Amersham (UK). Adriamycin was dissolved in reaction buffer (40 mM Tris, pH 8.0, 100 mM KCl, 3 mM $MgCl_2$ and 0.1 mM EDTA), [^{14}C]adriamycin dissolved in TE buffer to a concentration of 1 mM and all anthracycline derivatives were dissolved in DMF. All drug solutions were stored at −20°C. Nicotinamide adenine dinucleotide (reduced) (NADH) was obtained from Pharmacia. Xanthine oxidase (specific activity 1.07 units/mg protein) was purchased from Calbiochem.

Sequenase (T7 DNA polymerase), M13 mp18 single-strand DNA and the −40 universal sequencing primer were obtained from United States Biochemical (Ohio), as components of the Sequenase kit. Calf thymus DNA was from the Worthington Biochemical Corporation, NJ.

Methods

The plasmid pSP64 was grown in *E.coli* JM101 cells and harvested using routine methods (24). For [^{14}C]adriamycin studies, the plasmid was linearised with the blunt end generating enzyme, *Pvu*II. The 497 bp fragment (containing the lac UV5 promoter) used for transcription studies was isolated from pRW1 as described previously (22).

[^{14}C]Adriamycin–DNA binding studies. In a typical time course reaction, linear pSP64 (25 μM bp) was reacted with [^{14}C]adriamycin (10 μM) in transcription buffer comprising 40 mM Tris, pH 8.0, 100 mM KCl, 7 mM DTT, 3 mM $MgCl_2$ and 0.1 mM EDTA. The reaction was performed both in the presence and absence of 40 μM Fe(III) ions and incubated at 37°C for up to 48 hr. The DNA was then extracted twice with Tris-buffered phenol, once with chloroform and the DNA precipitated in the presence of glycogen as an inert carrier. The pellet was washed, lyophilised and resuspended into 30 μl of TE buffer and 1 ml of OptiPhase 'Hisafe' 3 scintillation cocktail was then added. The incorporation of [^{14}C]adriamycin into the DNA fragment was determined by scintillation counting of the DNA on a Wallac 1410 Liquid Scintillation counter.

Xanthine oxidase studies. Xanthine oxidase was prepared for use by diluting 50 μl of the enzyme in a total of 2 ml reaction buffer and concentrating the solution back to the original volume by filtration with a Centricon 10 filter. The enzyme was subsequently used within 24 hr of the exchange of buffers. In time course experiments using the enzyme reducing system, the linearised DNA was reacted with [^{14}C]adriamycin in buffer devoid of DTT and Fe(III) ions but replaced by 4 mM NADH and 0.5 unit/ml of xanthine oxidase. Following incubation of the drug with the enzymatic reducing system for up to 60 hr, aliquots were removed and the unbound drug extracted and the DNA assayed for drug binding as described above.

In vitro transcription. An initiated transcription complex was formed from the 497 bp fragment as described previously (25). The initiated transcription complex was reacted with 20 μM of each of the various anthracyclines in the presence of 40 μM Fe(III) ions for 24 h prior to the addition of elongation nucleotides. The reactions were terminated and the samples denatured, subjected to electrophoresis and the gel dried and autoradiographed as described previously (22). Band quantitation was performed using a Molecular Dynamics Model 400B PhosphorImager and ImageQuant software (Molecular Dynamics, CA).

Detection of adriamycin adducts on single-strand DNA. The M13-40 universal primer was 5′-end labelled by polynucleotide kinase in the presence of [γ^{32}P]ATP using routine methods (24). M13 mp18 single-strand DNA (450 μM nuc) was reacted overnight with 20 μM adriamycin in buffer containing 40 mM Tris, pH 8.0, 100 mM KCl, 7 mM DTT, 3 mM $MgCl_2$ and 0.1 mM EDTA. No Fe^{3+} was included as this resulted in extensive cleavage of the ssDNA. Control reactions were performed in the absence of adriamycin. The reacted DNA was subsequently annealed to the labelled primer at 65°C for 2 min, cooled to room temperature and then Sequenase added. The primer was subsequently extended through the template by the addition of 2 mM of all four nucleotides and 50 mM NaCl to yield a high molecular weight, radioactively labelled nascent DNA strand. Dideoxy sequencing reactions were performed as recommended in the Sequenase kit manual. The reactions were terminated by the addition of formamide loading buffer and the samples denatured at 75°C for 2 min and subsequently quenched on ice prior to loading onto an 12% denaturing polyacrylamide gel. Extended electrophoresis was performed for 4 hr at 2000 V and 45 mA before the gel was fixed, dried and autoradiographed. Band quantitation was performed using a Molecular Dynamics Model 400B PhosphorImager and ImageQuant software (Molecular Dynamics, CA).

pH dependence. Calf thymus DNA (170 ml, 25 μM bp) was reacted with 10 μM adriamycin, and 75 μM Fe(III) in 40 mM Tris and the pH adjusted to 5–8 as required. Complete removal of unreacted and intercalated drug was achieved by two phenol extraction followed by one chloroform extraction (23). The DNA was then precipitated with ethanol and redissolved in 2 ml of transcription buffer, pH 8.0. The amount of adducts was then quantitated from the visible absorbance of the non-extractable drug component at 508 nm.

RESULTS

We have shown previously from *in vitro* transcription studies that the formation of adriamycin-induced adducts with DNA is dependent on the reaction time and bothdrug and Fe(III) concentrations (22). From this work the optimised reaction conditions were a reaction time of 24 h (37°C) with 10 μM adriamycin, 50 μM (nucleotide) DNA, and 75 μM Fe(III) in a transcription buffer at pH 8. More recently we have shown that adducts continue to form for reaction times up to 50 h under these conditions (23). For these studies intercalated and unreacted drug were removed by a phenol extraction procedure (23) and the extent of formation of adducts then quantitated from the remaining visible absorbance (or radioactivity when using ^{14}C-labelled drug). These established conditions and procedures were generally maintained but one component of the reaction was varied at a time in order to establish the effect of that parameter on the extent of formation of adducts.

Temperature

The temperature of the reaction was varied from 10–55°C. Virtually no adduct was formed at 10°C but was enhanced approximately 40-fold at 40–50°C when in the presence of Fe(III), but only 4-fold in the absence of Fe(III) (Figure 1A). All previous reactions had been performed at 37°C and this was therefore maintained as the default temperature since this resulted in a near maximal level of adduct formation.

Reducing agent

Adriamycin-induced adducts were originally detected using a buffer required for an *in vitro* transcription footprinting assay (21,22) and this buffer has continued to be used to form these adducts. This buffer required the presence of 2–10 mM of DTT in order to stabilise the RNA polymerase. To assess whether DTT contributed to the formation of adducts, the concentration was varied from 0–10 mM in the reaction mixture and subjected to a 24 h reaction time at 37°C. A low level of adduct wasdetected in the absence of DTT but was enhanced some 20-fold in the presence of 8–10 mM DTT (Figure 1B), with approximately 90% of the maximal level resulting at 5 mM DTT. In the absence of Fe(III) there was no dependence on adduct formation, and only the background level was observed even at 10 mM DTT.

To examine whether there was an absolute requirement for DTT as a reducing agent, alternative reducing agents were used at the same concentration (7 mM) and the level of adduct quantitated for each reaction system. The amount of adduct detected using β-mercaptoethanol was approximately 10% of the level achieved with DTT, and was double that of the control reaction lacking any reducing agent (Table 1).

Buffer

The transcription buffer contained several components specifically required for optimal initiation and elongation of the transcription process (BSA, EDTA, Mg(II), etc.). All of these extraneous components were removed and adducts formed in a simple buffer consisting of just 40 mM Tris, pH 8.0, 7 mM DTT and 75 μM Fe(III). The same amount of adducts were formed in this simple buffer system, indicating that none of the components removed contributed to the formation of adducts. There was a pronounced dependence of adduct formation on pH (Table 2). Maximal adducts were detected at pH 7 and this was reduced by approximately 60% and 35% at pH 6 and 8 respectively.

DNA

Adduct formation was also dependent on the DNA concentration. The amount of adduct formed after a 24 h reaction was proportional to DNA concentration up to 15 μM bp, and reached a plateau level above 25 μM bp (Figure 1C). Comparison to the continuing build-up of adducts past 24 h observed previously with 25 μM DNA bp (23) therefore indicates that saturation of adduct sites on the DNA was not the limiting factor under those conditions. In the absence of Fe(III) the amount of adduct was greatly reduced, as noted previously (23).

Oxygen

The role of oxygen in the formation of adducts by adriamycin was investigated since the chemistry of the reduction of adriamycin is known to vary with the oxygen level (26–28). The labelled drug was reacted with DNA to form adducts under both aerobic and anaerobic conditions and the results are

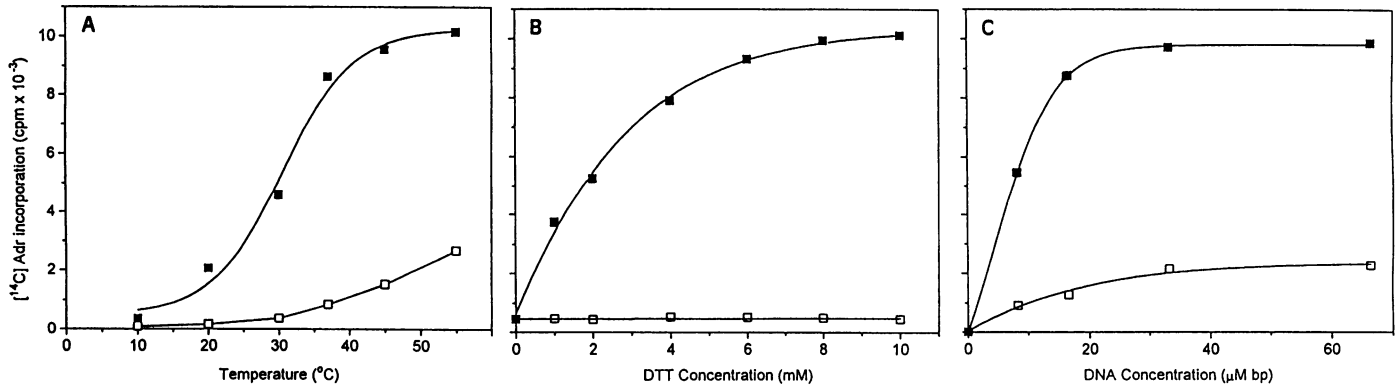


Figure 1. Dependence of adduct formation on reaction conditions. [^{14}C]Adriamycin (10 μM) was routinely reacted with linearised pSP64 DNA (25 μM bp) in reaction buffer (40 mM Tris, pH 8.0, 100 mM KCl, 3 mM $MgCl_2$, 7 mM DTT and 0.1 mM EDTA) in the presence (■) or absence (□) of 40 μM Fe(III) ions for 48 h at 37°C, except for Panel **A** (24 h reaction at 10–55°C), Panel **B** (48 h reaction, 0–10 mM DTT) and Panel **C** (48 h reaction, 0–70 μM DNA bp). At the completion of the reaction the samples were extracted twice with Tris-saturated phenol and once with chloroform and the DNA ethanol-precipitated in the presence of glycogen. The DNA was resuspended in TE buffer and 1 ml of scintillation fluid added and the radioactivity incorporated into the DNA measured by liquid scintillation counting.

summarised in Table 3. Under both conditions adduct formation was low in the absence of Fe(III) but was increased 6–7-fold in the presence of Fe(III). There was little effect of oxygen in the presence of Fe(III), with only a 20% increase of adduct yield in an oxygenated environment compared to anaerobic conditions.

Adriamycin derivatives

A range of adriamycin derivatives (Figure 2) were reacted for 24 h with a 497 bp fragment of DNA containing the lac UV5 promoter. The DNA was then subjected to *in vitro* transcription and the ability of each derivative to form adducts was quantitated from the intensity of blockage induced at position 37, the first GpC site encountered by RNA polymerase (22). The relative ability of each derivative to induce adducts at that site was then normalised with respect to the amount observed for Adriamycin, and these relative values are summarised in Figure 2.

Only four derivatives exhibited a similar capacity to that of Adriamycin to form adducts, and these were daunomycin, daunomycinol, adriamycinol and the 6-deoxy,4-demethoxydaunomycin (**2**–**5**). Two other derivatives (**6**, **7**) exhibited a clear but reduced ability to form adducts (approximately 50% of that of adriamycin) while one (**8**) showed only a minimal capacity to induce adducts.

Reduction by xanthine oxidase

To examine if the mechanism for formation of adriamycin-induced adducts involved the reduction of adriamycin, the drug was reacted with DNA in the presence of xanthine oxidase and NADH, a system known to be able to reduce adriamycin (17,29,30) and also known to yield DNA adducts with other quinione-based drugs such as mitomycin c (31). Increasing amounts of adducts were detected with increasing reaction time (Figure 3A). No adducts were detected under these conditions in the absence of the enzyme.

Since xanthine oxidase is known to contain residual Fe(III) (32) it was necessary to confirm that the adducts arose from the enzymatic activity of the drug and not merely from the effect of Fe(III) associated with the enzyme. This was accomplished by varying the level of the cofactor (NADH) in the reaction mixture. An increasing amount of NADH resulted in an increasing level of adduct, confirming that NADH was required for formation of the adduct and that the process of adduct formation was mediated by an enzymic process requiring NADH as cofactor (Figure 3B).

Single-strand DNA

Since adducts were readily formed with dsDNA, and the mechanism of formation appeared to involve a reductively activated species which then reacted with appropriate centres on dsDNA, it was likely that they would also react with ssDNA.To minimise the possibility of duplex structures arising from hairpin helices, or from complementary regions on reannealed sections of DNA from denatured mammalian sources, the ssDNA of choice for this work was from the virus M13. The M13 DNA was reacted with [^{14}C]adriamycin as for dsDNA and unreacted drug extracted with phenol in an identical manner. Increasing amounts of adducts were detected with increasing reaction time (Figure 4).

The sequence-specificity of adducts formed with ssDNA was established using a primer-extension assay. In this procedure adducts were first formed on the ssDNA, an end-labelled primer then annealed to the ssDNA and DNA synthesis subsequently initiated with DNA polymerase. Elongation of the primer proceeds until blocked at adduct sites and the blocked lengths of newly synthesised DNA were then separated on a sequencing gel and visualised by use of a PhosphorImager (Figure 5).

Elongation of the oligonucleotide primer by Sequenase was extremely efficient on the untreated M13 template, and only a small amount of background pausing is evident. The C and G sequencing lanes were also very clear and devoid of any significant background pausing. The drug-treated M13 template however yielded twenty one groups of blockages which could be resolved sufficiently to permit a quantitative analysis of relative intensity at each nucleotide (Figure 6). The major characteristic of these sites is that the Sequenase is blocked 1-3 nucleotides prior to most guanine residues on the M13 template reacted with adriamycin. This is also true for all eight of the high intensity sites (arbitrarily taken as greater than 2% of the total blockage intensity in the sequence region shown in Figure 6) which afforded good sequence resolution (sites 3–7, 11, 13 and 14). The offset of the newly synthesised strand is generally one nucleotide prior to the adduct site and this isillustrated in Figure 6 where a sloping line has been used to connect every guanine residue of the reacted M13 strand with observed blockages.

Only two of the 21 blockage regions (12 and 21) are not associated with a nearby downstream guanine residue on the

Table 1. Dependence of adduct formation on reducing agent

Reducing agent	Number of adducts −Fe(III)	+Fe(III)
H_2O	1.3	1.3
β-mercaptoethanol (7 mM)	1.4	2.4
glutathione (7 mM)	1.3	3.0
DTT (7 mM)	1.5	13.3

The number of adducts was calculated from the incorporation of non-extractable [^{14}C]adriamycin into DNA, and is expressed as adducts per 1000 nucleotides.

Table 2. Effect of pH on adduct formation

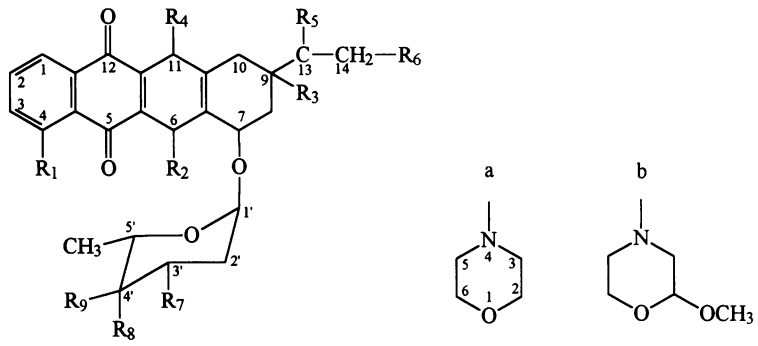
pH	Number of adducts A_{508}	^{14}C
5	4.2	7.7
6	20	11
7	38	50
8	19	33

The number of adducts was calculated from the amount of drug associated with DNA after a phenol extraction procedure, and is expressed as adducts per 1000 nucleotides. The adduct level was quantitated by absorbance at 508 nm, and also by scintillation counting when using [^{14}C]adriamycin.

Table 3. Effect of oxygen on adduct formation

Reducing system	Number of adducts $-O_2$	$+O_2$
DTT	5.2	6.9
DTT + Fe(III)	33.3	41.7

The number of adducts was calculated from the amount of non-extractable [^{14}C]adriamycin associated with DNA, and is expressed as adducts per 1000 nucleotides.



No.	Derivative	R1	R2	R3	R4	R5	R6	R7	R8	R9	Adduct Capacity[c]
1	Adriamycin	OCH_3	OH	OH	OH	O	OH	NH_2	OH	H	1.00
2	daunomycin	OCH_3	OH	OH	OH	O	H	NH_2	OH	H	0.82
3	daunomycinol	OCH_3	OH	OH	OH	OH	H	NH_2	OH	H	0.7[d]
4	adriamycinol	OCH_3	OH	OH	OH	OH	OH	NH_2	OH	H	0.7[d]
5	6-deoxy, 4-demethoxydaunomycin	H	H	OH	OH	O	H	NH_2	OH	H	0.67
6	N-trifluoroacetyladriamycin-14-valerate (AD32)	OCH_3	OH	OH	OH	O	$OCO(CH_2)_3CH_3$	$NHCOF_3$	OH	H	0.5[d]
7	11-deoxydaunomycinol	OCH_3	OH	OH	H	OH	H	NH_2	OH	H	0.39
8	4-demethoxy, 6-deoxy, 6-aminodaunomycin	H	NH_2	OH	OH	O	H	NH_2	OH	H	0.12
9	6-methoxydaunomycin	OCH_3	OCH_3	OH	OH	O	H	NH_2	OH	H	0.0
10	9-methoxydaunomycin	OCH_3	OH	OCH_3	OH	O	H	NH_2	OH	H	0.0
11	4-demethoxy, 3'-deamino, 4'-deoxy, 4'-epiaminodaunomycin	H	OH	OH	OH	O	H	H	H	NH_2	0.0
12	4-demethoxy, 3'-deamino, 3'-hydroxy, 4'-deoxy, 4'-epiadriamycin	H	OH	OH	OH	O	OH	OH	H	OH	0.0
13	3'-deamino, 3'-(4-morpholinyl)adriamycin	OCH_3	OH	OH	OH	O	OH	a	OH	H	0.0
14	3'-deamino, 3'-(2-methoxy-4-morpholinyl)adriamycin	OCH_3	OH	OH	OH	O	OH	b	OH	H	0.0

Figure 2. Relative adduct forming capacity of derivatives of adriamycin. Each derivative was reacted for 24 h with a 497 bp fragment of DNA containing the initiated lac UV5 promoter. The mole-fraction of blocked transcript was then ascertained for the first drug-induced blockage site, and then normalised with respect to the blockage observed for adriamycin. (**a**, **b**) The R7 substituent is defined by separate morpholino structures above. (**c**) Adduct capacity is the relative ability to form adducts with respect to that observed for adriamycin. (**d**) Estimated from transcriptional blockages on other gels.

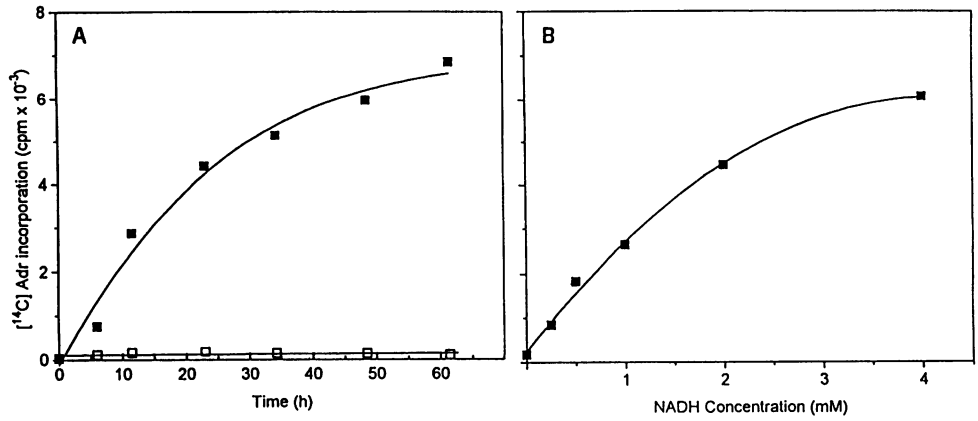


Figure 3. Dependence of xanthine oxidase/NADH-induced DNA adduct formation on reaction conditions. Dependence on reaction time (Panel **A**): DNA (25 μM bp) was reacted with adriamycin (10 μM) both in the presence (■) and absence (□) of NADH in buffer containing xanthine oxidase. The reaction was incubated at 37°C for 0–62 h. At various time intervals samples were removed, unbound drug extracted and the DNA samples measured for drug incorporation. Dependence on NADH concentration (Panel **B**): DNA (25 μM bp) was reacted with [^{14}C]adriamycin (10 μM) in buffer containing 0.5 U/ml xanthine oxidase and 0–4 mM NADH (but no DTT). The reactions were incubated at 37°C for 21 h. Non-bound drug was extracted and the drug incorporated into the DNA fragment measured by scintillation counting.

reacted DNA strand, and the cause of these blockages remains obscure.

DISCUSSION

The ability of xanthine oxidase to induce the formation of adriamycin adducts on DNA demonstrates the absolute requirement for a reducing system for the formation of adducts. It also establishes that the only role for DTT is that of a reducing agent, and that it is not a component of the adduct itself.

The extensive reaction time required to achieve maximal adduct levels following incubation with the enzymatic reducing system was similar to that of the DTT/Fe(III) system, and this suggests that once the drug has been reduced it is in an activated form, and the subsequent biomolecular reaction with either dsDNA or ssDNA is the rate-determining step. It is also significant that adducts form with both dsDNA and ssDNA because this implies that a duplex structure is not an inherent prerequisite for the formation of adducts.

The most likely way in which the DTT may serve as a reducing agent in this system is by direct interaction with Fe(III). The existence of a DTT-Fe complex is well documented (33). Since adriamycin is known to bind to Fe(III) with high affinity (2,28,34,35) the DTT−Fe−adriamycin complex offers a means of 1e− reduction of adriamycin, with Fe(III) serving the role of coupling the substrate (adriamycin) to the reducing agent (DTT). If the 1e− reduced species (semiquinone) is produced by this mechanism, then this can result in a series of reactions which result in the 2e− reduced species (hydroquinone) and this in turn can lead to the quinone methide (tautomeric with the C7 carbocation) which is capable of reacting with both nucleophiles and electrophiles (36). This reductive pathway has now been extensively documented for the anthracyclines and the existence of the quinone methide has now been confirmed (16). Although DTT has the same reduction potential as adriamycin (−0.33 V) (37,38) and would therefore be considered incapable of reducing adriamycin, it has recently been shown that chelation of adriamycin by Fe(III) causes the reduction potential of the drug to become more positive by 0.16 V and hence be more readily reduced (39).

The quinone methide has a half life of 15 s in aqueous solution where reaction with water (hydrogen abstraction) leads to 7-deoxyadriamycinone (36). If the drug is localised close to DNA, perhaps by means of its association with Fe(III) [Fe(III) has an affinity of 10^{12} M^{-1} for DNA (40)], then this may provide a mechanism for the enhanced yield of adduct with DNA than would otherwise be expected.

Drug structural requirements

Two major features are apparently required for adduct formation. Firstly, the C9 sidechain does not appear to be critical since there

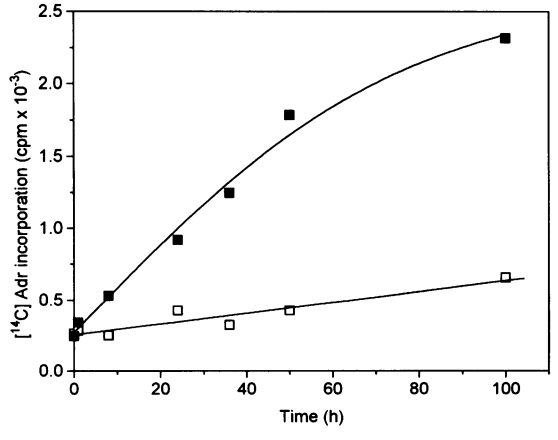


Figure 4. Formation of adriamycin-induced adducts with single-strand DNA. Adducts were formed as described in Materials and Methods for [^{14}C]adriamycin−DNA binding studies, but using 50 μM (nucleotide) of M13 DNA in the presence (■) and absence (□) of 10 μM Fe^{3+}.

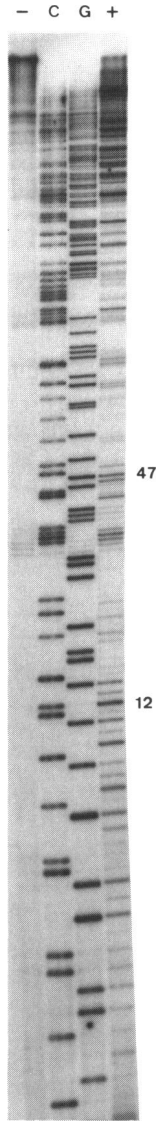


Figure 5. Sequence-specific adriamycin-induced adduct sites on single-strand DNA. M13 DNA was reacted with 20 μM adriamycin overnight in the absence of Fe^{3+}. The DNA was annealed with an end-labelled primer which was then extended with Sequenase. Extension of the primer was performed after treatment of the M13 DNA in the presence (+) or absence (−) of adriamycin. The C and G sequencing lanes were obtained using the untreated DNA in the presence of dideoxy CTP and dideoxy GTP respectively. The numbering system used is that employed in the Sequenase DNA sequencing kit protocol.

is no absolute requirement for a carboxyl at C13 (similar adduct levels were observed with both adriamycin and adriamycinol), nor a requirement for the hydroxyl at C14 (similar adduct levels for adriamycin and daunomycin). The C9 sidechain can also be modified extensively (as in AD32) yet still retain the capacity to form adducts. Secondly, there is no absolute requirement for the C11 hydroxyl (compare adriamycin and 11-deoxyadriamycinol). Although Fe(III) is known to chelate to the C11 and C12 oxygens (41,42) it is likely that at high Fe:adriamycin ratio, an alternative chelation site may involve the C5 and C6 oxygens. If this is the case, then as long as one of these chelation sites remains the Fe(III) binding capacity is retained—the effect of 6-deoxy or 11-deoxy derivatives is therefore likely to alter the site of chelation to the drug, but may not eliminate the capacity of the drug to be reduced and to form adducts.

Model of adducts

The most likely reactive sites on the drug for formation of an adduct are the C7 position and the C9 sidechain. Given the evidence that the adducts result from the reductive activation of the drug, the knowledge that such activation can lead to the formation of a quinone methide, and that such activated species have been shown to bind to nucleophiles (16), together with the apparent requirement for a guanine N^2 centre (21), the simplest interpretation is that the adducts comprise a (guanine N^2)−(7-deoxyaglycone) linkage. Such a linkage might be expected to exhibit some reversibility, as observed (23) because of the inherent reducibility of the anthracycline chromophore to yield a quinone methide. However, it should be noted that there is no direct evidence of such a linkage at this stage. Although there is good evidence that the C9 sidechain is reactive in adriamycin (but not in daunomycin) and results in the loss of the carbon at position 14 (43), this process does not appear to be involved in adduct formation since similar adduct levels were observed for adriamycin and daunomycin (Figure 2).

Although there is now a large body of information concerning the factors that affect the *in vitro* formation and stability of these adriamycin-induced adducts, thechemical composition of the adducts remains obscure. Because of the paucity of direct chemical evidence concerning the nature of the adducts it is not appropriate to speculate further at this stage on the chemical composition of the adduct. A complete understanding of these adducts must await mass spectral studies of the adduct itself, and this work is currently in progress.

CONCLUSIONS

The present work has demonstrated that adriamycin-induced adducts can be formed *in vitro*, and appear to involve a DTT/Fe reducing system. The mechanism of transfer of electrons from thiols to Fe-adriamycin has been well documented (41,44) and this process may also occur *in vivo* to some degree since it is now clear that adriamycin can sequester Fe(III) from ferritin (3,32,45). This activation may also be carried out in cells by one or more of a range of bioreductive enzymes, some of which are known to be localised in the nucleus (17,18,46). Although there have been several reports of the detection of adriamycin-induced adducts in cells (11−14) the adducts have yet to be isolated and characterised. While the adducts formed *in vitro* may not prove to be identical to those formed under cellular conditions, an understanding of the nature of these *in vitro* adducts will greatly assist future *in situ* and *in vivo* studies of these adducts.

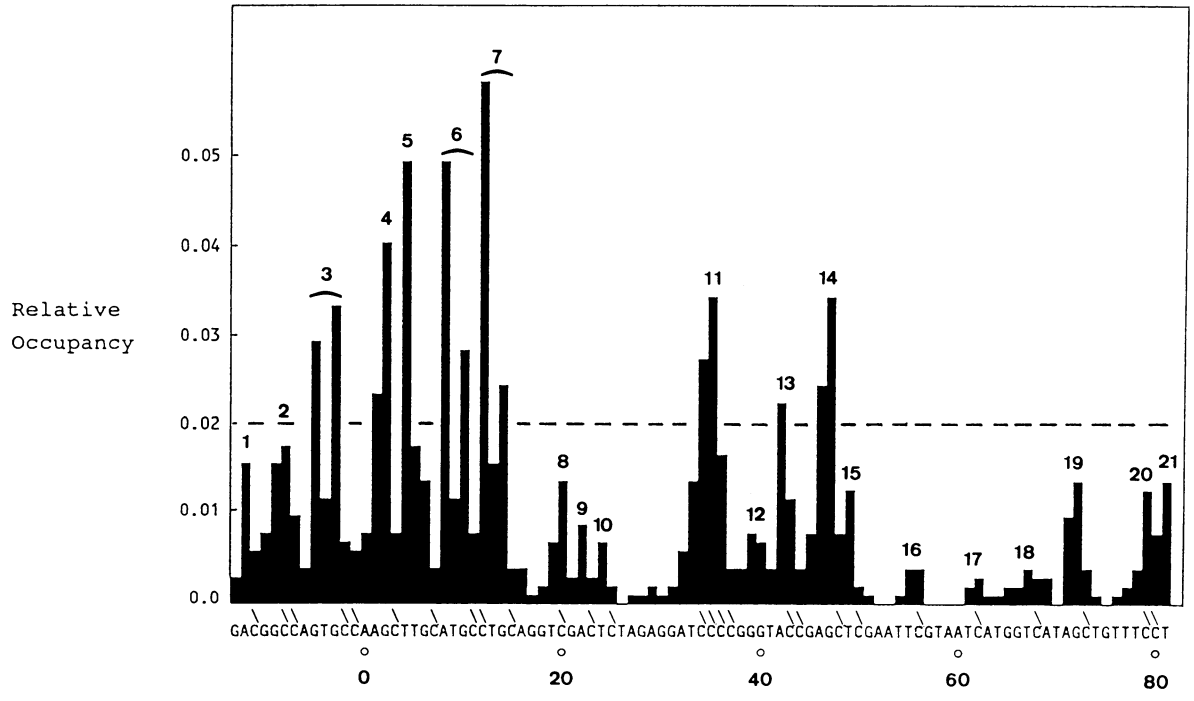


Figure 6. Relative occupancy of adducts. The relative intensity of each band shown in Figure 5 in the region −7 to 82 was quantitated and expressed as a fraction of the total blockage intensity in that region. High intensity sites are taken as those above 0.02 (i.e. 2% of total blockage intensity), and this cut-off is shown as a dashed line.

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REFERENCES

1. Weiss, R.B. (1992) *Seminars in Oncol.* **19**, 670–686.
2. Myers, C.E., Mimnaugh, E.G., Yeh, G.C. and Sinha, B.K. (1988) *Anthracycline and Anthracenedione–Based Anticancer Agents* (Lown, J.W. Ed.) Elsevier, Amsterdam, pp. 527–569.
3. Myers, C.E. (1992) *Cancer Chemother. Biol. Resp. Modifiers Ann.* **13**, 45–52.
4. Schneider, E., Hsiang, Y. and Liu, L.F. (1990) *Adv. Pharmacol.* **21**, 149–183.
5. Capranico, G. and Zunino, F. (1992) *Eur. J. Cancer* **28A**, 2055–2060.
6. Holm, C., Covey, J., Kerrigan, D., Kohn, K.W. and Pommier, Y. (1991) in *DNA Topoisomerases in Cancer* (Pomesil, M. and Kohn, K. Eds.) Oxford University Press, pp 161–171.
7. Binaschi, M., Capranico, G., De Isabella, P., Mariani, M., Supino, R. and Tinelli, S. (1990) *Int. J. Cancer* **45**, 347–352.
8. Gerwirtz, D.A. (1991) *Biochem. Pharmacol.* **42**, 2253–2258.
9. Del Bino, G. and Darzynkiewicz, Z. (1991) *Cancer Res.* **51**, 1165–1169.
10. Zwelling, L.A., Bates, E., Altschuler, E. and Mayes, J. (1993). *Biochem. Pharmacol.* **45**, 516–520.
11. Sinha, B.K. and Sik, R.H. (1980) *Biochem. Pharmacol.* **29**, 1867–1868.
12. Konopa, J. (1990) *Pharmacol. Ther.* **7** (suppl), 83–94.
13. Bartoszek, A. and Wolf, C.R. (1992) *Biochem. Pharmacol.* **43**, 1449–1457.
14. Cummings, J., Willmott, N., Hoey, B.M., Marley, E.S. and Smyth, J.F. (1992) *Biochem. Pharmacol.* **44**, 2165–2174.
15. Egholm, M. and Koch, T.D. (1989) *J. Am. Chem. Soc.* **111**, 8291–8293.
16. Gaudiano, G., Frigerio, M., Bravo, P. and Koch, T.H. (1990) *J. Am. Chem. Soc.* **112**, 6704–6709.
17. Pan, S.-S. and Bachur, N.R. (1980). *Mol. Pharmacol.* **17**, 95–99.
18. Sinha, B.K., Trush, M.A., Kennedy, K.A. and Mimnaugh, E.G. (1984) *Cancer Res.* **44**, 2892–2896.
19. Fisher, J., Abdella, B.R.J. and McLane, K.E. (1985) *Biochemistry* **24**, 3562–3571.
20. Cummings, J., Bartoszek, A. and Smyth, J.F. (1991) *Analyt. Biochem.* **194**, 146–155.
21. Phillips, D.R., White, R.J. and Cullinane, C. (1989) *FEBS Lett.* **246**, 233–240.
22. Cullinane, C. and Phillips, D.R. (1990) *Biochemistry* **29**, 5638–5646.
23. Cullinane, C., van Rosmalen, A. and Phillips, D.R. (1994) *Biochemistry*, in press.
24. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning. A Laboratory Manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
25. Skorobogaty, A., White, R.J., Phillips, D.R. and Reiss, J.A. (1988) *Drug Design and Delivery* **3**, 125–152.
26. Sinha, B.K. (1989) *Chem.-Biol. Interactions* **69**, 293–317.
27. Cummings, J., Anderson, L., Willmott, N. and Smyth, J.F. (1991) *Eur. J. Cancer* **27**, 532–535.
28. Gianni, L. and Myers, C.E. (1992) *Cancer Treatment and the Heart* (Muggia, F.M., Green, M.D. and Speyer, J.L., Eds.) The John Hopkins University Press, Baltimore, pp. 9–46.
29. Kappus, H. (1986) *Biochem. Pharmacol.* **35**, 1–6.
30. Bates, D.A. and Winterbourn, C. (1982) *Biochem. J.* **203**, 155–160.
31. Tomasz, M., Chowdary, D., Lipman, R., Shimotakahara, S., Veiro, D., Walker, V. and Verdine, G.L. (1986) *Proc. Natl. Acad. Sci. USA* **83**, 6702–6706.
32. Vile, G.F. and Winterbourn, C.C. (1986) *J. Free Rad. Biol. Med.* **2**, 393–396.
33. Van Dyke, M.W. and Dervan, P.B. (1983) *Nucleic Acids Res.* **11**, 5555–5567.
34. May, P.M., Williams, G.K. and Williams, D.R. (1980) *Eur. J. Cancer* **16**, 1275–1276.
35. Garnier-Suillerot, A. (1988) in *Anthracycline and Anthracendione–based Anticancer Agents* (Lown, J.W., Ed.) Elsevier, Amsterdam, pp. 129–161.
36. Kleyer, D.L. and Koch, T.D. (1983) *J. Am. Chem. Soc.* **105**, 5155–5156.
37. Cleland, W.W. (1964) *Biochemistry* **3**, 480–485.
38. Land, E.J., Mukherjee, T., Swallow, A.J. and Bruce, J.M. (1983) *Arch. Biochem. Biophys.* **225**, 116–121.
39. Dikalov, S.I., Rumyantseva, G.V., Piskunov, A.V. and Weiner, L.M. (1992) *Biochemistry* **31**, 8947–8953.
40. Netto, L.E.S., Da Costa Ferreira, A.M. and Augusto, O. (1991) *Chem.-Biol. Interactions* **79**, 1–14.
41. Myers, C.E., Gianni, L., Simone, C.B., Klecker, R. and Greene, R. (1982) *Biochemistry* **21**, 1707–1713.
42. Gelvan, D. and Samuni, A. (1988) *Cancer Res.* **48**, 5645–5649.
43. Gianni, L., Vigano, L., Lanzi, C., Niggeler, M. and Malatesta, V. (1988) *J. Natl. Cancer Inst.* **80**, 1104–1111.
44. Myers, C.E., Gianni, L., Zweier, J., Muindi, J., Sinha, B.K. and Eliot, H. (1986) *Fed. Proc.* **45**, 2792–2797.
45. Demant, E.J.F. and Norskov-Lauritsen, N. (1986) *FEBS Letts.* **196**, 321–324.
46. Bartoszek, A. and Wolf, C.R. (1992) *Biochem. Pharmacol.* **43**, 1449–1457.