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Characteristics of the Binding of the Anticancer Agents Mitoxantrone and Ametantrone and Related Structures to Deoxyribonucleic Acids†

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ABSTRACT: The binding constants for interaction of the anticancer agents mitoxantrone and ametantrone and several congeners with calf thymus DNA and the effects of ionic strength changes have been determined spectrophotometrically. The agents show a preference for certain sequences, particularly those with GC base pairs, and the magnitude of the specificity depends on the specific substituents on the anthraquinone ring system. The binding constant for mitoxantrone with calf thymus DNA in 0.1 M Na^+, pH 7, is approximately 6×10^6 M^{-1}, and the rate constant for the sodium dodecyl sulfate driven dissociation of mitoxantrone from its calf thymus DNA complex under the same solution conditions and 20 °C was determined to be 1.3 s^{-1}. The unwinding angle of mitoxantrone determined independently by viscosity measurements and by a novel assay employing calf thymus topoisomerase shows excellent agreement for a value of 17.5°. The viscosity increase of sonicated calf thymus DNA varies considerably with the substituent on the anthraquinone ring system. Binding studies employing T4 and ϕw-14 DNAs in which the major groove is occluded and the reverse experiment with anthramycin-treated calf thymus DNA indicate at least part of the mitoxantrone molecule may lie in the minor groove.

Although doxorubicin (adriamycin) shows wide-spectrum activity against a range of human malignancies (Arcamone, 1978, 1981), its clinical efficacy is currently limited by the severe risk of irreversible cardiac damage (Bonadonna, Monfardini, 1969; Lenaz et al., 1976; Smith, 1969). Consequently intensive efforts have been undertaken both to understand the underlying molecular origin of the cardiotoxicity (Doroshow et al., 1980; Lown et al., 1982; Goodman & Hochstein, 1977) and thereby to obviate or minimize it by structural modification (Tong et al., 1979) or by the rational design and de novo synthesis of less toxic drugs (Lown et al., 1981). Among the more promising drugs developed are the anthracene derivatives 1,4-dihydroxy-5,8-bis[[2-[(2-hydroxyethyl)amino]ethyl]amino]-9,10-anthracenedione [mitoxantrone (**1**); Figure 1] and its congeners (Murdock et al., 1979).

Biochemical evidence suggests that, in common with the anthracyclines, nucleic acids are among the principal cell targets of these drugs and that they cause inter alia profound changes in chromatin structure including compaction (Traganos et al., 1980; Waldes & Center, 1982; Bowden et al., 1982; Citarella et al., 1982). Although the experience with other clinically useful anticancer drugs would suggest parallel modes of action involving other cellular macromolecules (Lown, 1983), there are strong indications that interaction of mitoxantrone with cellular DNA contributes significantly to the cytotoxic action. However, the exact nature of the DNA interactions is at present unclear. We recently reported electron microscopy evidence for the intercalative binding of mitoxantrone and bis[(4,5-dihydro-1*H*-imidazol-2-yl)-hydrazone] 9,10-anthracenedicarboxaldehyde (bisantrene) to different DNAs and the phenomenon of inter-DNA interactions and association produced by mitoxantrone and certain congeners (Lown et al., 1984). Since the unique shape of these drugs apparently prevents intercalation of all parts of the chromophore in contrast to doxorubicin (Neidle, 1978), we report an examination of the substituent dependence in the binding of mitoxantrone and its congeners to DNA. In addition, we report the measurement of their binding constants to DNA and the kinetics of dissociation together with a determination of the unwinding angle by two independent methods and a determination of the groove and base preference for DNA binding in order to characterize the DNA binding of this important class of anticancer agents.

Materials and Methods

Materials

Compounds. Mitoxantrone, ametantrone, and their congeners (**1–9**) (Figure 1) were supplied by Dr. K. C. Murdock of Lederle Laboratories, Pearl River, NY. Their synthesis, purification, and properties have been described previously (Murdock et al., 1979). Daunorubicin was purchased from

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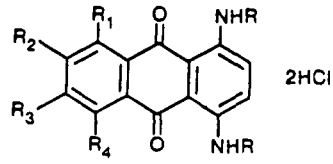

#	R	R_1	R_2	R_3	R_4
1 (mitoxantrone)	$-(CH_2)_2NH(CH_2)_2OH$	OH	H	H	OH
2 (ametantrone)	$-(CH_2)_2NH(CH_2)_2OH$	H	H	H	H
3	$-(CH_2)_3NH(CH_2)_2OH$	OH	H	H	OH
4	$-(CH_2)_2NHCH_2CHOHCH_3$	OH	H	H	OH
5	$-(CH_2)_2NH_2$	OH	H	H	OH
6	$-(CH_2)_2N(CH_3)H$	OH	H	H	OH
7	$-(CH_2)_2N(CH_3)_2$	OH	OH	H	H
8	$-(CH_2)_2N(CH_3)_2$	H	H	H	H
9	$-(CH_2)_2N(CH_3)_2$	H	H	$-CH_3$	H

FIGURE 1: Structures for mitoxantrone (**1**), ametantrone (**2**), and congeners.

Boehringer-Mannheim and was found to be pure by thin-layer chromatography (TLC),[1] NMR, and UV–visible spectral analysis. Ethidium bromide was purchased from Sigma Chemical Co., St. Louis, MO.

Buffers. PIPES buffers, used for the spectrophotometric determination of drug–DNA binding, contained 10 mM PIPES, 1 mM EDTA, and sodium chloride as follows: PIPES 10, 0.1 M NaCl; PIPES 20, 0.2 M NaCl; PIPES 50, 0.5 M NaCl. All were adjusted to pH 7.0. Tris-HCl buffer at pH 8 was employed for the topoisomerase determination of unwinding angle.

DNA Samples. Calf thymus DNA was from Worthington, and *Micrococcus lysodeikticus* and *Clostridium perfringens* were from Sigma. DNA samples were prepared for use by dissolving 5–10 mg/mL DNA in PIPES 50 with overnight stirring at 4–5 °C. The viscous solution was briefly sonicated several times with intermediate stirring at 4–5 °C to assist in dissolving the DNA. This solution was then saturated with N_2 gas and sonicated for 2 h under N_2 at 0–5 °C with stirring. A Heat Systems Ultrasonics W375B pulsed sonicator was used. The sonicator was set at a 10% pulse duty cycle with power adjusted to keep the temperature between 0 and 5 °C. The sample in a glass tube or beaker was suspended in an ethanol–water bath at −10 to −15 °C for cooling. A microthermistor probe connected to a YSI Model 42 telethermometer was kept in the sample during sonication to constantly monitor temperatures. After sonication, the sample was centrifuged and passed through a 0.22-μm Millipore filter to remove particulates. The sample was then phenol extracted, under the high-salt conditions, to remove any protein contamination. Typically, no protein precipitate was seen at the interface after the second phenol extraction. At least two extractions were conducted after no protein was visible at the interface. We have found, however, that the *M. lysosdeikticus* DNA contains a significant amount of protein before phenol extraction. After the phenol extractions the samples were ether extracted to remove phenol, and the DNA was precipitated with 2 volumes of ethanol at 5 °C. The precipitate was collected by centrifugation, dried, redissolved, and extensively dialyzed against the appropriate PIPES buffer. Samples were analyzed by T_m, hypochromicity, electrophoresis, NMR, and UV–visible spectroscopy. DNA polymer samples were purchased from P-L Biochemicals. These samples were dissolved in and dialyzed against the appropriate PIPES buffer. They were analyzed by the methods indicated above.

Phage PM2 covalently closed circular (CCC) DNA was prepared according to the procedure of Pulleyblank & Morgan (1975a,b) except that, instead of concentrating the phage in the crude lysate by dextran sulfate, the Millipore Minitan apparatus with 100 000-Da cutoff membrane was found not only to concentrate the phage more rapidly but also to give higher yields of CCC-DNA (30–50 OD_{260} units/L). The DNA was additionally purified by ethidium bromide–cesium chloride density gradient centrifugation.

T4 DNA and λ-DNA (M_r 31 × 10^6) were obtained from Miles Laboratories Inc., Elkhart, IN, and φw-14 DNA was a gift from Dr. D. Scraba, Department of Biochemistry, University of Alberta.

Enzymes. Calf thymus DNA topoisomerase was purified up to the Sephadex G-100 step (Pulleyblank & Morgan, 1975a,b) and further purified on a Sephadex G-75 column. Only those fractions free of any DNA binding proteins as determined by the pH 8 ethidium bromide assay (Morgan et al., 1979) were pooled for the drug unwinding determinations. The topoisomerase was shown to relax PM2 CCC-DNA completely by giving a drop in fluorescence of 33 ± 3% in the pH 12 ethidium bromide assay (Pulleyblank & Morgan, 1975a,b) which corresponds to a superhelical density of −0.126 given an average ethidium unwinding angle of 28° per intercalated cation (Wang, 1974).

Methods

Spectrophotometric Methods. Absorbance measurements in the UV–visible region were made on a Cary 219 spectrophotometer interfaced to an Apple IIe microcomputer through a bidirectional digital communications port. Cell holders were thermostated by using Neslab or Haake circulating water baths. Wavelength scans and extinction coefficient measurements were made in cells from 1- to 10-cm path length at the wavelength or wavelength range appropriate for the compound being investigated. Extinction coefficients of compounds bound to DNA were determined at the same wavelength as the extinction coefficient measurements of the free compound, but a larger molar excess of DNA was present ([DNA-P/[compound] > 100). The Cary 219 was also used in spectrophotometric binding studies. To remove some of the random error, for each absorbance measurement in the absence or presence of DNA, the microcomputer calculated the average of 100 acquired absorbance readings at the preselected wavelength for the compound under study. These averaged absorbance values were converted by the microcomputer to ν (moles of compound bound per mol of DNA base pairs) and free ligand concentrations using the free and bound extinction coefficients for the compound (Wilson & Lopp, 1979). At the end of the titration, the computer plotted the digitized data which was in the fraction-bound range 0.2–0.8. Any binding results outside of this range are subject to large systematic errors due to experimental errors in extinction coefficients (Wilson & Loop, 1979). The computer then calculated nonlinear least-squares best-fit K (equilibrium constant) and n (base pairs per binding site) values (and optionally the cooperativity parameter ω if selected by the operator) from the site exclusion method of McGhee & von Hippel (1974).

Dissociation Kinetics. SDS-driven dissociation kinetics experiments were conducted by using the method of Müller

[1] Abbreviations: TLC, thin-layer chromatography; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); EDTA, ethylenediaminetetraacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Da, dalton; SDS, sodium dodecyl sulfate.

& Crothers (1968) as previously described (Chanadrasekaran et al., 1984; Wilson et al., 1985). Equal volumes (0.1 mL) of a drug–DNA complex in PIPES 10 buffer were mixed with a 1% SDS solution in PIPES buffer adjusted to the same sodium ion concentration as PIPES 10 with NaCl. Stopped-flow mixing was performed with an Aminco-Morrow instrument adapted to a Johnson Foundation MB-2 spectrophotometer. Output from the spectrophotometer was fed to an OLIS 3820 data acquisition system. Two hundred data points in a preselected time range could be fitted by using from one to three exponentials with the software supplied with the OLIS system. Typically, several kinetics runs were stored on disk and averaged to improve signal to noise before the fitting was done.

Viscosity. Viscometric measurements were made in Cannon-Ubbelhohde semimicro dilution viscometers using electronic timing as previously described (Jones et al., 1980). Sonicated calf thymus DNA described above was used for measuring length increases of linear DNA. Unwinding angle measurements were conducted by using closed circular supercoiled Col E_1 DNA prepared as previously described (Jones et al., 1980).

Determination of DNA Unwinding Angle Employing Topoisomerase. The reaction conditions used for determining the mitoxantrone unwinding angle by the topoisomerase method (Lee & Morgan, 1978) were 10 mM Tris-HCl, pH 8, 2 mM EDTA, 0.1 M NaCl, 0.17 mM PM2 CCC-DNA (P), and varying concentrations of the drug (0 up to 60 μL of a 0.15 mM solution) in a total volume of 100 μL at 37 °C. When the topoisomerase was present, it was added in amounts that relaxed PM2 CCC-DNA under the reaction conditions completely in 10 min. However, aliquots (30 μL) were taken at 0.5, 1, and 3 h to ensure relaxation in the presence of the drug. The samples were added to 2 mL of the alkaline ethidium assay solution (Mogan et al., 1979). Fluorescence readings were taken both before and after heat denaturation (96 °C/4 min followed by rapid cooling) by using a standard such that 10 μL of calf thymus DNA at 1.0 A_{260} gave 50 fluorescence units.

Results

Determinations of DNA Unwinding Angle by Mitoxantrone. (*i*) *Viscosity Assay.* The results of several viscometric titrations of closed circular supercoiled DNA with mitoxantrone are shown in Figure 2. These titrations reveal the classical uncoiling, viscometric maximum at the open circular form, and reverse coiling of the supercoiled DNA on titration with compounds that cause unwinding of the double helix (Revet et al., 1971). By use of analogous titrations with an appropriate standard that has a known unwinding angle, the average unwinding angle of the double helix per bound molecule can be calculated from a Vinograd plot of the results from Figure 2. When ethidium is used, with a 28° unwinding angle as the standard, a linear fit to the unwinding results (inset, Figure 2) gives an unwinding angle of 17 °C for mitoxantrone in PIPES 10 buffer. Unwinding measurements at the same ionic strength in MES buffer at pH 6 and Tris buffer at pH 8 gave similar unwinding angles.

(*ii*) *Topoisomerase Assay.* As may be seen in Figure 3 different concentrations of drug are added to a fixed amount of PM2 CCC-DNA (plotted on the abscissa as the ligand to DNA base pair molar ratio ν) in two series, one with and one without topoisomerase. Topoisomerase will relax the negatively supercoiled CCC-DNA in the absence of drug, and the topological winding number α will increase. However, at a critical level of drug, just sufficient to relax PM2 CCC-DNA,

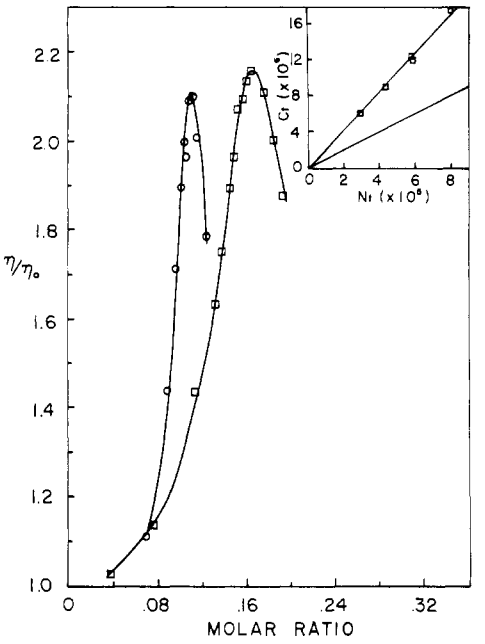

FIGURE 2: Viscometric titrations of closed circular supercoiled Col. E_1 DNA with mitoxantrone (□) and ethidium (○). Titrations were conducted in PIPES 10 buffer at 30 °C at DNA base pair concentrations of 7.0×10^{-5} M for mitoxantrone and 6.0×10^{-5} M for ethidium. Several titrations at different DNA concentrations were conducted for each intercalator, and the intercalator C_T and DNA base pair concentration at the peak position in the titration were plotted (inset) to determine the intercalator unwinding angle. The slope of the linear plot gives the moles of drug per DNA base pair required to unwind the DNA supercoils. The results for mitoxantrone (□) with a linear least-squares best fit line are shown with the ethidium results indicated only by a line for reference.

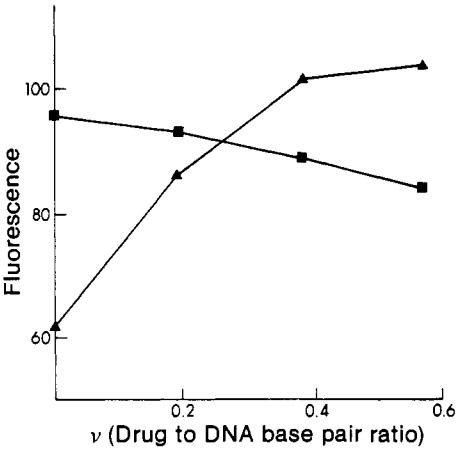

FIGURE 3: Determination of unwinding angle for mitoxantrone employing calf thymus topoisomerase using PM2 DNA. The ordinate F indicates the fluorescence reading in arbitrary units in the pH 12 ethidium assay. $D/P = \nu$ is the molar mitoxantrone (drug) to DNA phosphate ratio. Reactions were carried out as under Materials and Methods. Reactions with topoisomerase (▲); reactions without topoisomerase (■). This represents the 30-min sampling. Very similar curves were obtained at 1 and 3 h.

the topoisomerase will have no effect on α. At higher levels of drug the topoisomerase will relax positive supercoils and thus decrease α. Therefore, when almost saturating amounts of ethidium are used and since ethidium binds in an amount governed by α, the enhanced fluorescence of ethidium when intercalated into DNA can be used to determine the critical level of drug used to just relax the CCC-DNA. Such a level will be indicated by the crossover of the curves with the line obtained when topoisomerase is omitted for the various drug concentrations. There are two considerations with this ap-

proach to determining the unwinding angle: (i) The dissociation constant for the drug/DNA complex at the crossover point must be known. In the case of mitoxantrone the association constant for DNA is of the order of 10^6 M^{-1} under the reaction conditions (0.1 M NaCl) so that the drug is essentially completely bound (>99%). (ii) The topoisomerase must be free of any appreciable contaminants (e.g., histones) that might bind the DNA and introduce local supercoiling (e.g., in nucleosomes). This was found to be a problem with calf thymus topoisomerase on long-term storage which had lost much of its activity. When the topoisomerase was used at a higher level, DNA binding contaminants interfered with the determination of the unwinding angle. This problem was not recognized previously (Lee & Morgan, 1978), and therefore, it is stressed that before any experiment the topoisomerase should be shown to give the maximum loss of fluorescence of ~33% for complete relaxation of PM2 CCC-DNA and be free of DNA binding proteins (see Materials and Methods).

As shown in Figure 3 mitoxantrone gives a well-defined crossover for the curves generated by (+)- and (−)-topoisomerase. The decrease in fluorescence for the reaction mixture containing topoisomerase relative to that without in the absence of drug showed the expected decrease in fluorescence of 31% with an estimated 5% error. For mitoxantrone the crossover point for the three time points averaged at a ν ratio of 0.264. This corresponds to an unwinding angle of 17.5° (with 5% variation for the three time points) and in excellent agreement with the value determined independently by viscometry.

In the absence of topoisomerase a slight negative curve is obtained with increasing mitoxantrone concentration, showing the drug competes with the ethidium even when the latter is present in large excess in the fluorescence assay.

As a further control the unwinding angle of ethidium was also determined by the same method assuming a superhelix density of −0.126 for PM2 CCC-DNA. The crossover point gave a ν ratio off 0.150 or a corresponding unwinding angle of 29°, which is within experimental error of 28°. Thus, the results are internally consistent. In a previous report (Lee & Morgan, 1978) the crossover point was given at a ν ratio of 0.11 rather than 0.150. The source of this error is probably due to the use of a less purified topoisomerase at a concentration that contained DNA binding proteins which interfered. Under the conditions used essentially all the drug, ethidium, was bound at the crossover for a binding constant of 6×10^5 M^{-1} (Le Pecq et al., 1967).

Viscosity Assay: *Sonicated DNA*. All of the compounds of Figure 1 cause an increase in sonicated DNA viscosity as expected for intercalators. Results for compounds showing the widest range of viscosity effects are illustrated in Figure 4 along with results for daunorubicin for reference. As can be seen, mitoxantrone, **3**, and daunorubicin gave quite similar viscometric titration curves. The viscosity increase with ametantrone is somewhat less, and the increase in viscosity for **9** is exceptionally small. All other compounds of Figure 1 produce viscosity increases between those of mitoxantrone and ametantrone.

Spectrophotometric Results. A spectrophotometric titration of mitoxantrone with DNA is illustrated in Figure 5. Mitoxantrone, **3**, and daunorubicin give isobestic behavior in PIPES 10, 20, and 50 buffers. We could, however, obtain isosbestic behavior for some of the compounds of Figure 1 only in PIPES 50 buffer (of the three buffers listed above), and for this reason PIPES 50 was chosen as a standard system for binding comparisons. The loss of isosbestic behavior for the

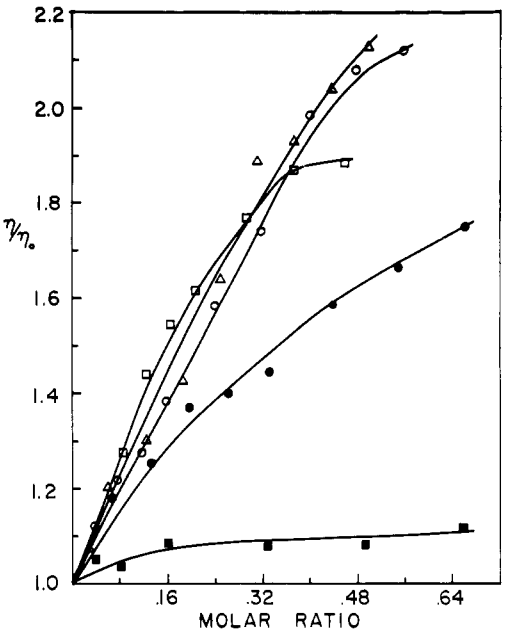

FIGURE 4: Sonicated DNA changes as a function of drug: DNA base pair ratio for (□) daunorubicin, (○) mitoxantrone (**1**), (▲) **3**, (●) ametantrone (**2**), and (■) **9**. Experiments were conducted in PIPES 10 buffer at DNA base pair concentrations of (6–7) × 10^{-5} M.

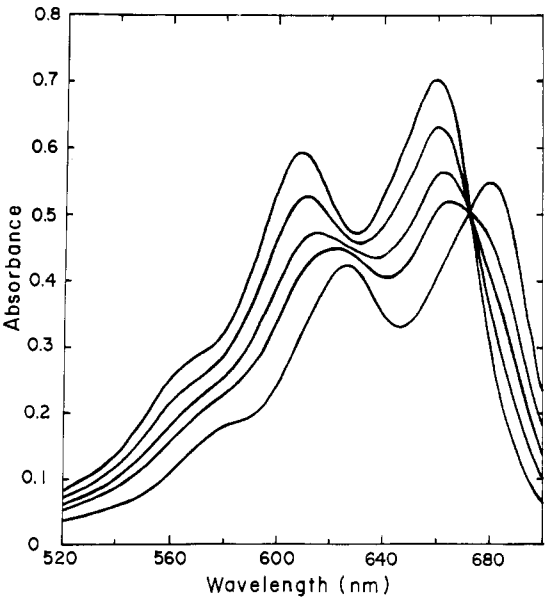

FIGURE 5: Spectrophotometric titration of mitoxantrone with calf thymus DNA in PIPES 10 buffer. The mitoxantrone concentration was 3.2×10^{-6} M, and the ratio of mitoxantrone to DNA base pairs for the curves from top to bottom in the figure are 0, 1.2, 0.6, 0.4, and 0.14. There is little change in the spectrum on further decreasing the ratio.

compounds of Figure 1 was directly related to the removal of hydroxy groups. The compounds with no hydroxy groups on the side chain were, by far, the most difficult with which to work. They presumably have some reasonably favorable competing binding mode (e.g., outside stacking) which is more sensitive to ionic strength than the primary binding mode. Compounds, like ametantrone, without the aromatic ring hydroxy groups but with the side-chain hydroxy groups were less inclined to display the secondary binding mode. It should be noted that, at low ionic strength (below 0.1) and/or higher concentration, mitoxantrone also loses isosbestic behavior.

Binding Affinity: *Ionic Effects*. Equilibrium constants were determined for several of the compounds of Figure 1 as a

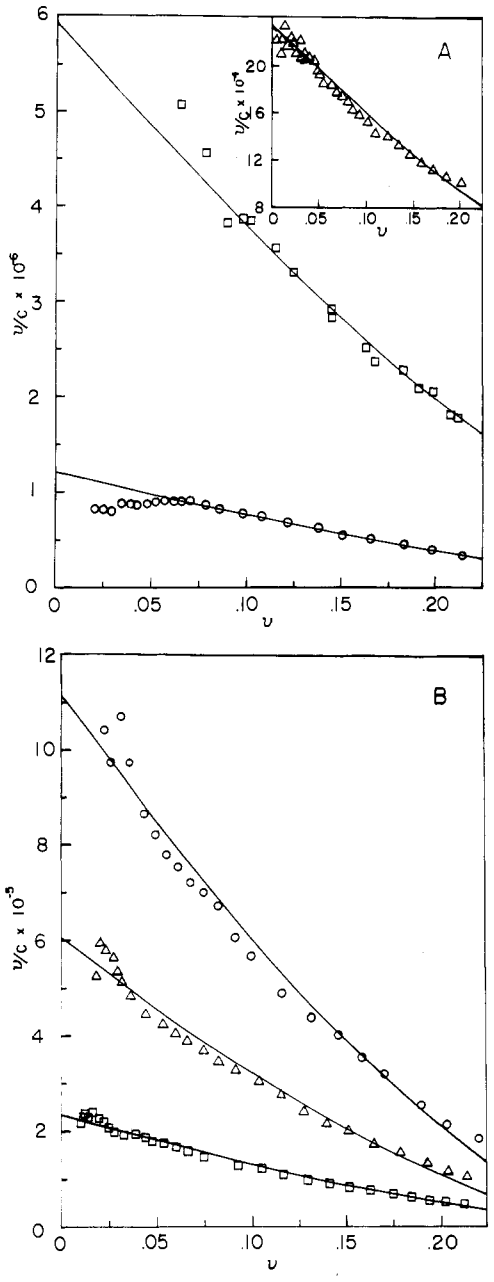

FIGURE 6: Spectrophotometric titration results for calf thymus DNA in PIPES 10, 20, and 50 with (A) **3** and (B) daunorubicin. Points are shown from titrations, and the curves are nonlinear best-fit lines according to eq 1. The best-fit n values for **3** were between 2.2 and 2.4 and between 2.9 and 3.0 for daunorubicin. (A) PIPES 10 (□); PIPES 20 (○); PIPES 50 (△). (B) PIPES 10 (○); PIPES 20 (△); PIPES 50 (□).

function of ionic strength by using the spectrophotometric method described under Materials and Methods. Scatchard plots of some of the results are shown in Figures 6 along with lines drawn from best-fit K and n values from eq 1 (McGhee

$$\nu/c = K[1 - n\nu][(1 - n\nu)/[1 - (n - 1)\nu]]^{n-1} \quad (1)$$

& von Hippel, 1974) where c is the free intercalator molarity. Mitoxantrone and **3** have similar variations in the binding constants over the range of ionic strengths investigated, 0.1–0.5. Scatchard plots for the binding of **3** in PIPES 10, 20, and 50 are shown in Figure 6A and can be compared to Scatchard plots for daunorubicin in the same buffers which are shown for reference in Figure 6B. Standard log K vs. log [Na^+] plots for these results are shown in Figure 7. The slope is approximately 2 for the plot of the binding constants for **3** and

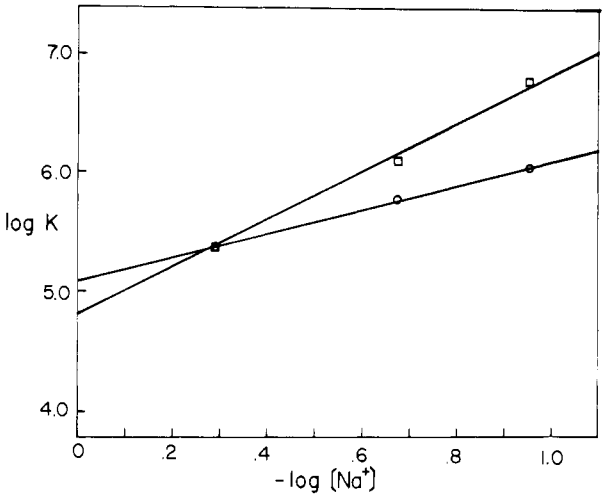

FIGURE 7: log K values from Figure 6 are plotted as a function of −log [Na^+]: (□) **3** and (○) daunorubicin.

Table I: Spectrophotometric Binding Results

compound	K ($\times 10^{-4}$)[a]	n[a]
daunorubicin	23.3	2.8
mitoxantrone (**1**)	17.8	2.9
3	23.5	2.2
4	18.1	2.0
5	10.9	2.3
6	10.3	2.7
ametantrone (**2**)	1.6	[b]
7	3.0	[b]
8	2.5	[b]
9	2.3	[b]

[a] K and n values in PIPES 50 buffer were determined by a nonlinear least-squares fit of eq 1 to binding isotherms as described under Materials and Methods. [b] Because of the significantly reduced binding constant of these compounds, accurate data could be obtained only at low binding ratios, and the error in determining n values was large. For compounds **8** and **9**, which have no hydroxy groups, very erratic behavior was obtained at the higher binding densities.

is one for the daunorubicin plot in Figure 7 as expected for di- and monocations (Wilson et al., 1985). At low ionic strength the binding of **3** to DNA is greater than daunorubicin. At 0.5 M Na^+ they are approximately equal, and daunorubicin should bind more strongly at ionic strengths above 0.5 (Table I). At physiological conditions the mitoxantrone analogues with four hydroxy groups (two aromatic and two side chain) bind more strongly to DNA than daunorubicin. The SDS-driven dissociation rate constant for dissociation of mitoxantrone from its calf thymus DNA complex was determined to be 1.3 s^{-1}. This rate constant is in the same range as the dissociation rate constants observed for anthracycline–DNA dissociation (Gabbay et al., 1976) but is much lower than the constants observed for other common intercalations such as ethidium (Ryan & Crothers, 1984; Chandrasekaran et al., 1984). A more detailed analysis, as a function of ratio of mitoxantrone to DNA base pairs, currently in progress, indicates that the dissociation reaction actually consists of at least two exponential decay curves.

Binding Affinity: Molecular Effects. Since all of the compounds of Figure 1 give isosbestic points in PIPES 50 when titrated with DNA, this buffer was used for directly comparing the binding of these compounds to DNA. Best-fit K and n values were determined as described above and are collected in Table I. The compounds fall into three groups based on their equilibrium constants: (1) mitoxantrone, **3**, and **4**, which have the same hydroxy substitution pattern on the aromatic

Table II: Base Pair Specificity in Binding

DNA type	K ($\times 10^{-5}$)[a]	n[a]
Mitoxantrone		
poly[d(G-C)·d(G-C)]	47.5	2.4
M. lysodeikticus	23.9	2.8
calf thymus	10.4	2.4
C. perfringens	7.7	2.6
poly[d(A-T)·d(A-T)]	12.9	2.6
Compound **7**		
M. lysodeikticus	7.9	2.4
calf thymus	5.8	2.1
C. perfringens	4.7	2.2

[a] K and n values in PIPES 20 buffer were determined by a nonlinear least-squares fit of eq 1 to binding isotherms as described under Material and Methods.

ring and which have side-chain hydroxy groups, have binding constants near 2×10^5; (2) **2**, **5**, and **6**, which have the same ring hydroxy substituents but no side-chain hydroxy groups, have binding constants near 1×10^5; (3) **8** and **9**, which have no ring or side-chain hydroxy groups, and **7**, which has different substituent positions for the ring hydroxy groups and no side-chain hydroxy groups, have binding constants near 0.2×10^5. Because of complications, such as outside binding modes, it is much more difficult to determine accurate n than K values (note Figure 6). From the fitting standpoint K is more sensitive to results in low binding density region while n is more dependent on results at higher binding densities. A general value of n of 2.5 ± 0.5 is in the range typically observed for intercalators and is essentially all that can be quantitatively determined for these compounds.

Base Pair Specificity. The binding of mitoxantrone to DNA samples of varying base pair composition has been investigated by the spectrophotometric method, and the results are shown in Figure 8. PIPES 20 was chosen as the buffer for this comparison since it allows a wide range of equilibrium constants to be accurately determined in the region of interest for this compound. Best-fit K and n values were determined by using eq 1 as above. These values are collected in Table II and were used to construct the solid lines in Figure 8. Several facts are obvious from these results. First, mitoxantrone binds strongly to all of the DNA samples with the lowest binding constant being near 10^5 M^{-1} at this salt concentration. Second, there is a general preference for G,C base pairs in mitoxantrone binding, but there may also be some DNA sequence preference to the interaction since the binding to poly[d(A-T)·d(A-T)] is greater than for calf thymus and *C. perfringens* DNA samples which have a higher G·C content than the polymer. Finally, given the error limit in determining n, as indicated above, no significant differences in the base pairs per binding site for mitoxantrone on the different DNA samples can be seen. Results for binding of **7** to the three natural DNA samples are also collected in Table II. It can be seen that removing the hydroxy substituents from mitoxantrone results in a significant reduction in the G·C pair binding preference. Once again the n values for this compound and the different DNA samples are the same within experimental error.

Groove Specificity. The binding of mitoxantrone to T4 DNA [in which the major groove is occluded by α-glucosylated cytosine residues (Erickson & Szybalski, 1964)] and ϕw-14 DNA [in which the major groove is obstructed by spermidine (Kropinski et al., 1973)] was examined in order to test for a groove preference. The binding constants K determined by the ethidium equilibration procedure (Morgan et al. 1979) for mitoxantrone with T4 DNA and ϕw-14 were 2.7×10^6 M^{-1} and 1.33×10^6 M^{-1}, respectively, and compared with that of calf thymus DNA under comparable conditions of 2.5×10^6 M^{-1}.

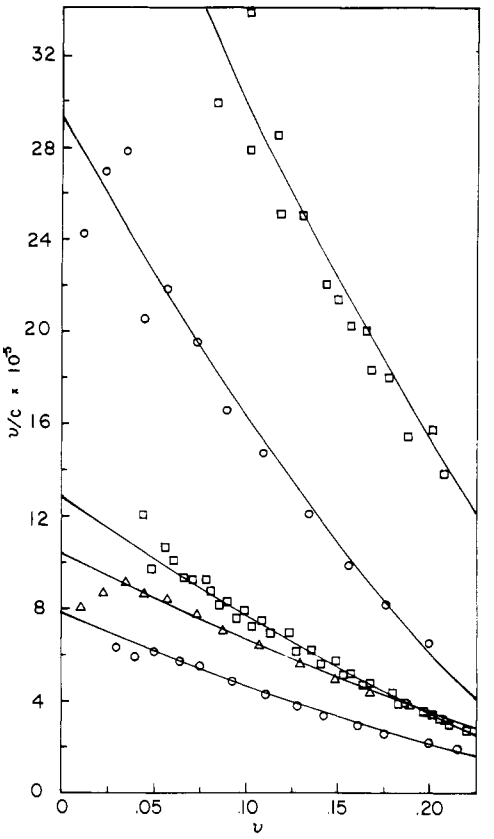

FIGURE 8: Scatchard plots for the binding of mitoxantrone to different DNA samples in PIPES 20 buffer: (□) poly[d(G·C)-d(G·C)]; (○) *M. lysodeikticus* DNA; (□) poly[d(A·T)-d(A·T)]; (△) calf thymus DNA; (○) *C. perfringens* DNA; from top to bottom in the figure. The points are from the spectrophotometric titration, and the curves are the best-fit K and n values by using eq 1. K and n values are collected in Table II.

By contrast the binding constants for the interaction of mitoxantrone with calf thymus DNA containing 0.035 anthramycin per phosphate and 0.1 anthramycin per phosphate were 1.4×10^6 M^{-1} and 0.5×10^6 M^{-1}, respectively.

Discussion

Widespread interest in the agents mitoxantrone and ametantrone and their congeners has arisen because of their clinical potential as anticancer agents (Citarella et al., 1982) and, more particularly, because of their apparent lower risk of cardiotoxic effects compared with the naturally occurring anthracyclines doxorubicin and daunorubicin (Citarella et al., 1982). There is compelling evidence that nucleic acids represent one of the principal cell targets in the mode of action of these agents nBowden et al., 1982; Citarella et al., 1982; Murdock et al., 1982) and that the binding of the drugs to DNA accounts, at least in part, for their cytotoxic properties (Foye et al., 1982; Kapuscinski et al., 1981). The exact nature of the modes of binding has, however, been controversial. Although mitoxantrone bears the requisite planar aromatic chromophore recognized as an essential requirement for intercalation (Lerman, 1961), the presence of the two extended side chains on positions 1 and 4 precludes incorporation of all parts of the molecule. This has led to various suggestions for alternative modes of binding (Kapuscinski et al., 1982; Foye et al., 1982). The form of the binding isotherm indicates at least two modes of binding presumed to include intercalation and exterior

electrostatic binding (Kapuscinski et al., 1982; Foye et al., 1982).

Our recent electron microscopy studies confirmed, by the characteristic extension in length of different DNAs, the intercalation of both mitoxantrone and bisantrene (Lown et al., 1984). Considerations of the observed G,C base preference for non-nearest-neighbor binding of mitoxantrone and inspection of the known sequence of pBR322 used for the electron microscopy studies suggested that the available intercalation sites are occupied and that additional external electrostatic binding of the drug also occurs. An estimate was made that PM2 DNA bound ca. 1700 mitoxantrone molecules per circle (10 300 base pairs). The present independent evidence from absorption spectral determinations of binding constants confirm that mitoxantrone and its congeners bind to duplex DNA with K values in the range $(2.35–0.16) \times 10^6$ M^{-1} at 0.5 M Na^+ and confirms the preference for binding to G,C sequences. Decreasing the ionic strength of the medium also causes an increase in binding of some of the drugs (Figure 6A,B) as expected (Record et al., 1976, 1978; Wilson & Lopp, 1979). Similar effects on the binding of daunorubicin to DNA have been seen by Chaires et al. (1982).

The agreement between the two values of the unwinding angle for mitoxantrone determined independently by viscosity measurements and with a topoisomerase is good. The value of 17.5° may be compared with that of ethidium at 28° and is consistent with the view of partial intercalation impeded by the two side chains. Alternatively the anthraquinone ring system may bind in a novel manner, parallel to the long axis of the base pairs, with one cationic substituent in each groove and with a reduced unwinding angle.

Evidence from X-ray diffraction studies on drug–oligodeoxyribonucleotide complexes indicates that for most antitumor agents examined to date that groups providing electrostatic binding extend into and are bound in the minor groove (Neidle, 1978; Arcamone, 1981). The present results confirm mitoxantrone binds strongly to T4 and ϕw-14 DNAs in which the major grooves are known to be partially blocked by α-glucosylcytidine residues (Erickson & Szybalski, 1964) and polyamines (Kropinski et al., 1973), respectively. However, mitoxantrone binds less effectively to anthramycin-treated calf thymus. Anthramycin is known to bind to the minor groove of DNA (Hurley, 1977). Although such observations should be viewed with caution, especially when dealing with relatively small molecules, these results are in accord with the view that at least part of the mitoxantrone molecule is accommodated in the minor groove of B-DNA.

The studies also suggest there is in the case of mitoxantrone an unusual sensitivity of binding to the substitution pattern and coplanarity of ring A. This expectation is borne out with the observation that for 2,3-dihydromitoxantrone in which the conformation of ring A approaches that of a half-chair (i.e., noncoplanar with rings B and C) the K is only 0.6 of that for mitoxantrone itself and by the significantly reduced viscosity increase of **9** with linear DNA (Figure 4).

In conclusion the present studies are in accord with the model for mitoxantrone involving substantial intercalation of the chromophore with some contacts of the side chains in the DNA grooves and preferential binding at G,C sites causing an unwinding angle of 17.5°. The physical evidence also suggests that there is an additional external binding component.

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Registry No. **1**, 65271-80-9; **2**, 64862-96-0; **3**, 73542-16-2; **4**, 70788-93-1; **5**, 96555-65-6; **6**, 96555-66-7; **7**, 69837-16-7; **8**, 69895-68-7; **9**, 70945-54-9.

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Detection of Neocarzinostatin Chromophore–Deoxyribose Adducts as Exonuclease-Resistant Sites in Defined-Sequence DNA†

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ABSTRACT: A 5′-end-labeled DNA restriction fragment was treated with the nonprotein chromophore of neocarzinostatin under anoxia in the presence of dithiothreitol, conditions known to maximize formation of chromophore–deoxyribose adducts. Under conditions where unmodified DNA was digested to completion, chromophore-treated DNA was highly resistant to digestion by exonuclease III plus the 3′ → 5′ exonucleolytic activity of T4 DNA polymerase and partially resistant to digestion by exonuclease III plus snake venom exonuclease. The electrophoretic mobilities of the products of exonucleolytic digestion suggested that (i) digestion by exonuclease III or T4 polymerase terminated one nucleotide before the nucleotide containing the adduct, (ii) the remaining nucleotide directly adjacent to the adduct (3′ side) could be removed by snake venom phosphodiesterase, but at a slow rate, (iii) the covalently linked chromophore decreased the electrophoretic mobilities of the digestion products by the equivalent of approximately three nucleotides, and (iv) adducts formed under anaerobic conditions occurred at the same nucleotide positions as the strand breaks formed under aerobic conditions (primarily at T and, to a lesser extent, A residues). The close similarity in sequence specificity of adducts and strand breaks suggests that a common form of nascent DNA damage may be a precursor to both lesions. A chromophore-induced free radical on C-5′ of deoxyribose, subject to competitive fixation by addition reactions with either oxygen or chromophore, is the most likely candidate for such a precursor. The base specificity of adduct formation does not reflect the reported base specificity of neocarzinostatin-induced mutagenesis, suggesting that lesions other than adducts may be responsible for at least some neocarzinostatin-induced mutations, particularly those occurring at G·C base pairs.

At least four types of lesions are formed in DNA by the antitumor antibiotic neocarzinostatin (or its isolated nonprotein chromophore), in the presence of sulfhydryl cofactors. The predominant lesion is a strand break, resulting from selective, oxygen-dependent oxidation of deoxyribose to a 5′-aldehyde (Kappen et al., 1982). The next most frequent lesion is release of free base, presumably associated with some as yet undetermined form of sugar oxidation (Kappen & Goldberg, 1983). In addition, at least two types of adducts between DNA and the nonprotein chromophore of neocarzinostatin have been detected. In the presence of 2-mercaptoethanol and oxygen, a labile adduct containing an oxidized deoxyribose is formed, although with a very low yield (Povirk & Goldberg, 1982a,b). In the presence of dithiothreitol, a much more stable adduct species is produced, with a somewhat higher yield (Povirk & Goldberg, 1984). Under anaerobic conditions, the yield of this type of adduct is further increased (while strand breakage and base release are suppressed), so that stable adducts become the predominant DNA lesion. This stable adduct species, and probably the labile adduct species as well, involves covalent linkage of the chromophore to deoxyribose in DNA.

Chromophore–DNA adducts were originally isolated as two or three base long fragments after extensive nucleolytic digestion of chromophore-treated DNA (Povirk & Goldberg, 1982a, 1984). The resistance of these chromophore–oligonucleotide adducts to further nuclease digestion suggested the possibility that adducts might, like pyrimidine dimers, block exonucleolytic digestion of DNA (Setlow et al., 1964). The sequence specificity of adduct formation could then be determined by mapping adducts as exonuclease termination sites in defined-sequence DNA (Tullius & Lippard, 1981; Royer-Poroka et al., 1981). We sought to compare the sequence specificity of adduct formation with that of strand breakage, as well as with the specificity of neocarzinostatin-induced mutagenesis (Foster & Eisenstadt, 1983).

Experimental Procedures

Preparation of Labeled Restriction Fragments. DNA of plasmid pMC1 (obtained from E. Eisenstadt) was isolated and digested with *Hin*cII. The 789 and 935 base pair (bp) fragments, containing sequences of the *lacI* gene (Calos et al., 1978), were separated on a 5% polyacrylamide gel and electroeluted. The 789-bp fragment was 5′-^{32}P end labeled by the exchange reaction (Maxam & Gilbert, 1980) and digested with *Dde*I, and the resulting 66-bp fragment was isolated on an 8% polyacrylamide gel. The sequence of the labeled strand of this fragment is 5′-AACCACCATC$_{10}$AAACAGGATT$_{20}$TTCGCCTGCT$_{30}$GGGGCAAACC$_{40}$AGCGTGGACC$_{50}$GCTTGCTGCA$_{60}$ACTCTC. A fragment containing the first 49

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