Investigations into the sequence-selective binding of mithramycin and related ligands to DNA

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ABSTRACT

The preferred binding sites for mithramycin on four different DNA fragments have been investigated by DNAase I footprinting. Sites containing at least two contiguous GC base pairs are protected by the antibiotic, the preferred binding site consisting of the dinucleotide step GpG (or CpC). Related antibiotics chromomycin and olivomycin produce similar, but not identical footprinting patterns suggesting that they can recognize other sequences as well. All three antibiotics induce enhanced rates of enzyme cleavage at regions flanking some of their binding sites. These effects are generally observed in runs of A and T and are attributed to DNA structural variations induced in the vicinity of the ligand binding site. The reaction of dimethylsulphate with N7 of guanine was modified by the presence of mithramycin so that we cannot exclude the possibility that these antibiotics bind to DNA via the major groove.

INTRODUCTION

Mithramycin (Figure 1) is an antitumour antibiotic effective against a wide variety of experimental and human tumours [1]. Although the drug is highly toxic it has proved useful for treating patients with disseminated testicular carcinomas [2-4] and Paget's disease [5]. The antibiotic has been shown to inhibit DNA and RNA synthesis in vivo usually with some preference for the latter [6-8]. In vitro mithramycin binds to double-stranded DNA in the presence of a divalent cation such as Mg$^{2+}$ [9] and inhibits both RNA and DNA polymerase reactions [7,10-11]. The interaction with DNA has been shown to require the presence of a guanine base [10,12]. It has been suggested that the antibiotic is specific for the 2-amino group of guanine since chromomycin, a closely related antibiotic, fails to interact with polyD polydC whereas mithramycin strongly inhibits the function of poly(DAP-T) as a template for RNA synthesis [10,13]. The guanine specificity probably resides in the chromophore portion of the molecule since it is retained in derivatives lacking one or more of the sugar residues [12].

The preferred binding sites for mithramycin and related antibiotics
chromomycin and olivomycin on a heterogeneous DNA have recently been investigated using (methidiumpropyl-EDTA) iron II, [MPE Fe(II)] as a DNA cleaving agent [14]. These were shown to be a minimum of three base pairs long and contain at least two contiguous GC base pairs. The preferred sites on the 70 base pair fragment of DNA examined were (in decreasing affinity) 3'-GGG, CGA>GCG, GCC>GCA, CCT>GTC-5'. However, because of the multiplicity of binding sites and their apparent dependence on the nature of the flanking sequences it was not possible to unambiguously define the preferred binding sequence. In the present study we have employed the technique of DNAase I footprinting to investigate the binding of mithramycin, and related antibiotics, to four different DNA fragments in an attempt to define its precise sequence selectivity more accurately.

The mode of interaction of this class of antibiotics with DNA is uncertain, although intercalation seems unlikely since they fail to affect the sedimentation coefficient, viscosity or melting temperature of linear DNA [6] and have little effect on the supercoiling of closed circular DNA [15]. However, it has been suggested that chromomycin binds by partial intercalation [12]. Other workers have reported kinetic and viscometric evidence that mithramycin intercalates between DNA base-pairs in a magnesium dependent fashion [16]. In the present study we have used DNAase I as a footprinting agent since it is known to be sensitive to the precise local helical structure of DNA [17,18]. We have also employed DNAase II footprinting and the G specific reaction with dimethylsulphate to probe the precise nature of the binding mechanism.

MATERIALS AND METHODS
Antibiotics and enzymes

Mithramycin was a gift from Pfizer Inc. USA. Chromomycin A3 was purchased from Sigma and olivomycin was a gift from Prof. G.P. Gause, Academy of Medical
Stock solutions of each antibiotic were prepared by direct weighing and dissolved in 10mM tris-HCl, pH 7.5 containing 10mM NaCl. All antibiotic solutions were stored in the dark at +4°C. Deoxyribonuclease I (DNAase I) was obtained from Sigma and prepared as a 7200 units/ml stock solution in 0.15M NaCl containing 1mM MgCl₂. It was stored at -20°C and diluted to working concentrations immediately before use. Deoxyribonuclease II (DNAase II) was obtained from PL Biochemicals and prepared as a 200 units/ml stock solution in 10mM ammonium acetate, pH 5.6 containing 0.2mM EDTA and stored at -20°C. This enzyme solution was used without any further dilution.

DNA fragments

The 160 base-pair duplex tyr T DNA fragment from E. coli containing the tyrosine tRNA promoter together with its adjacent sequences was isolated and labelled as previously described [17-19]. Incubation with reverse transcriptase, dGTP and α[³²P]dCTP led to selective labelling of the 3' end of the top strand (Figure 2) whereas incubation with reverse transcriptase, dTTP and α[³²P]dATP led to selective labelling of the 3' end of the bottom strand.

The 166-base-pair pTyr2 fragment (Figure 5b) was cut from plasmid pMLB1048 between the unique EcoRI and BstEII sites and was a gift from Mrs C.M.L. Low. The BstEII end (top strand) was selectively labelled with dCTP and α[³²P]dTTP while the EcoRI end (bottom strand) was labelled with dTTP and α[³²P]dATP.

The 65-base-pair fragment containing the sequence GATCC, labelled at the 5'end, was a gift from Dr H.R. Drew and had been prepared as previously described [20].

The 119-base-pair Xbso fragment (Figure 4) was cut from plasmid pXbsl, which contains a single repeating unit of X. borealis somatic 5S DNA in plasmid pBR322 [21], between the unique Hind III site and the first Ava I site on the inserted DNA. Plasmid pXbsl was a gift from Dr D. Rhodes. The Ava I end (top strand) was labelled with dGTP, dCTP and α[³²P]dTTP; the Hind III end (bottom strand) was labelled with dGTP, dCTP and α[³²P]dATP.

DNAase I footprinting

Samples (3μl) of the labelled DNA fragments (9 pmoles in base pairs) were incubated with 5μl of antibiotic solution (final concentration 5-25μM) at 37°C for 30 mins then digested with 2μl of DNAase I dissolved in 20mM NaCl, 2mM MgCl₂, 2mM MnCl₂ to give a final enzyme concentration of 0.01 units/ml. 3μl aliquots were removed from the mixture after 1, 5 and 30 mins and the reaction stopped by adding 2.5μl of 80% formamide containing 0.1% bromophenol blue,
Figure 2. DNAase I footprinting of mithramycin on the 160-base-pair tyr T DNA fragment. (a) Autoradiograph of the DNAase I digest of the 3'-end labelled bottom strand. Time in minutes after the addition of enzyme is shown at the top of each gel lane. The track labelled "G" is a dimethyl sulphate–piperidine marker specific for guanine. (b) Differential cleavage plot for differences in susceptibility to DNAase I digestion in the presence of 10μM mithramycin. Positive values indicate enhancement, negative values blockage.

10mM EDTA and 1mM NaOH. Samples were heated at 100°C for at least 3 mins prior to electrophoresis.

DNAase II footprinting

Mithramycin–DNA reaction mixtures were incubated as described above, then digested with 2μl of DNAase II (final enzyme concentration) 40 units/ml. 3μl aliquots were removed after 1, 5 and 30 mins and the reaction stopped by freezing on dry ice. Samples were heated at 100°C in 80% formamide for 3 mins prior to electrophoresis.

Reaction with dimethylsulphate

The dimethylsulphate reaction specific for guanine was performed under standard conditions [22] in the presence of various antibiotics and 5mM MgCl₂.

Densitometry

Autoradiographs were scanned using a Joyce-Loebl microdensitometer to
produce profiles from which the relative intensity of each band was measured. For DNA fragments TyrT, pTyr2 and XbsI data are expressed as fractional cleavage \( f = \frac{A_i}{A_T} \) as previously described [18,19] where \( A_i \) is the area under band i and \( A_T \) is the sum of the intensity under all bands in any gel lane. When comparing different digestion patterns care was taken to ensure that the extent of digestion was similar and limited to 20-40% of the starting material, so as to minimize the incidence of multiple cuts. Data from this analysis are presented in the form of \( \ln (f_{\text{antibiotic}} - f_{\text{control}}) \), representing the differential cleavage at each band relative to that in the control. The results are presented on a logarithmic scale for the sake of convenience, positive values indicating an enhanced rate of cleavage, negative values representing protection from cutting. The data from the autoradiographs of the 65-base-pair fragment are presented as the logarithm of the probability of cleavage at each band and have been calculated according to the method of Lutter [23], correcting for any multiple cutting that might have occurred.

**RESULTS**

**DNAase I footprinting**

Typical DNAase I digestion patterns for the bottom strand of the 160-base pair tyr T fragment in the presence of mithramycin are shown in Figure 2a. The digestion was performed in the presence of varying amounts of mithramycin in order to characterize any intermediate states of antibiotic-induced protection. The pattern obtained with 2\( \mu \)M mithramycin is intermediate in protection between the control and 10\( \mu \)M. For example the bands between 100 and 120 are reduced in intensity in the 2\( \mu \)M track while they are completely blocked by 10\( \mu \)M mithramycin. However, increasing the ligand concentration beyond 10\( \mu \)M caused no further changes in the pattern. Each gel lane contains about 100 reasonably well resolved bands; these were analysed as described in the Methods section and the results along with those for the other labelled strand are presented in the form of a differential cleavage plot in Figure 2b. It is immediately apparent that the cleavage pattern in the presence of the antibiotic is substantially different from that of the DNA alone. Two regions protected by the antibiotic can be easily discerned and, as anticipated, are found in GC rich regions. It is also evident that the rate of cleavage at certain bonds is strongly enhanced relative to that in the control.

The two sites protected by the antibiotic are located around positions 75 and 110, and can be seen on both strands. An additional blockage site is apparent on the top strand around position 133 in a region which is poorly...
resolved on the bottom strand. It is not possible to assess the exact size of
the region protected by each bound ligand from this data alone, since the two
sites, centred about positions 75 and 110, contain long runs of G and C
residues and may represent several overlapping binding sites. Indeed the
inhibition of cleavage around these two sites extends over 8-10 and 25-30
base-pairs respectively.

In order to obtain more precise data concerning the exact mithramycin
binding site(s) footprinting experiments were performed with two other DNA
fragments, pTyr2 and Xbs1, which contain different arrangements of GC
residues. Patterns of DNAase I digestion for the top strand of the Xbs1
fragment in the presence of mithramycin, chromomycin and olivomycin are shown
in Figure 3 and the results for both DNA strands are presented as differential
cleavage plots in Figures 4a,b,c. It is readily apparent that the antibiotics
induce both blockages and enhancements in the rate of DNAase I cleavage and
that the three ligands produce slightly different patterns. For mithramycin
blockages are located near positions 43, 51, 61, 69, 77 and 82 all of which
are rich in GC base-pairs. The minimum protected region at each site is now
easily seen to be three or four base-pairs similar to the value previously
reported [14]. At each site the block is staggered across the two strands by
about 2-3 bonds towards the 3'end as previously observed with DNAase I
footprinting [17-19].

The blockages produced by olivomycin and chromomycin include all the
sequences protected by mithramycin but appear to extend into other regions as
well, thereby masking a number of the enhancements seen with mithramycin. This
is especially clear at positions 46 and 86 where the enhanced cutting induced
by mithramycin is no longer apparent, and these bonds are now part of
protected regions. These differences between the three antibiotics will be
considered further in the Discussion. In this DNA fragment all regions
protected by the drugs are associated with at least two contiguous GC base-
pairs while enhanced rates of cleavage are apparent in AT rich regions
flanking these binding sites.

A typical DNAase I digestion of pTyr2 DNA in the presence of the three
antibiotics is displayed in Figure 5a and a differential cleavage plot
calculated from the data with mithramycin is presented in Figure 5b. While the
cleavage pattern is clearly altered by the presence of this ligand, for
example witness the enhanced cleavage around position 30 and the blockage
around position 48, the effect of mithramycin is not impressive. Larger
changes are produced by olivomycin and chromomycin, for example the extra
blockage at positions 70 and enhancement around position 81. However, these blockages are weak compared with the much clearer protection produced on the other two DNA fragments. It appears that this particular piece of DNA does not offer the required sequences necessary for tight binding. What then is peculiar about this DNA sequence? Clearly the fragment contains several GpC and CpG dinucleotide steps to which the antibiotics fail to bind. However, we notice that the dinucleotide step CpC (or GpG) is underrepresented in this DNA fragment being found only at positions 48 and 98 both of which form weak sites of protection. Upon inspection of the differential cleavage plots obtained with tyr T and XbaI DNAs it can be seen that most of the strongly protected regions occur around this very dinucleotide. We can therefore tentatively identify the preferred binding site of mithramycin as containing the dinucleotide CpC (or GpG). Chromomycin and olivomycin also bind well to this sequence yet appear to have a greater capacity for recognizing other sequences. The precision with which we are able to identify the preferred binding sites of these antibiotics will be considered further in the Discussion.

Structural changes upon binding

As well as protecting from DNAase I cleavage in the regions already discussed these antibiotics induce an enhanced rate of cutting at certain bonds relative to that in the control. Do these enhancements reveal anything about their interaction with DNA? The regions where cleavage by the enzyme is enhanced are rich in A+T residues. For example, on the tyr T fragment the sequences ACTTTTAAT around position 29, ATTTGATGATGAT around position 86 and TAAAAA around position 128 are cut more efficiently in the presence of the antibiotic. In each case the enhanced cutting occurs adjacent to sites where mithramycin is bound, suggesting that the effect results from some distortion of the local DNA structure to a form more susceptible to cleavage, generated by the binding of the ligand. This has been confirmed by the results of footprinting experiments performed at various temperatures between 4°C and 60°C. The mithramycin induced inhibition of cleavage is found to be independent of temperature while subtle changes are observed in the enhanced regions. For example, the relative intensity of bands (84,85), (89,90) and (92,93) are reversed at elevated temperatures. The cleavage at these regions

Figure 3. DNAase I digestion patterns for the XbaI fragment with mithramycin (MIT), olivomycin (OLIV) and chromomycin (CHRO). The top strand of Figure 4 is labelled at the 3'-end. Time in minutes after the addition of enzyme is shown at the top of each gel lane. For each drug the first two lanes are with 2µM antibiotic, the second two with 10µM.
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Figure a: DNA sequence and electrophoretic mobility shift assay (EMSA) bands for probe a.

Figure b: DNA sequence and EMSA bands for probe b.

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Figure 4. Differential cleavage plots for the XbsI DNA fragment in the presence of (a) 10μM mithramycin, (b) 10μM chromomycin, (c) 10μM olivomycin.

is not normally temperature sensitive [17] suggesting that we are indeed observing a ligand-induced change in local DNA structure.

We have obtained further information of the ligand-induced enhanced rates of cleavage by studying the effect of mithramycin on the DNAase I digestion pattern of a 65-base-pair DNA fragment containing the sequence CgT11C, as shown in Figure 6. Previous studies with this DNA fragment have indicated that the (dC) runs possess a wide minor groove while the runs of (dT) have a narrow minor groove [20]. Both these regions are cut poorly on account of their deviation from the structure required for optimal enzyme activity [20]. As anticipated cleavage of the (dC) segment is reduced in the presence of mithramycin while pronounced enhancements are seen in the (dT) segment. A simple interpretation of this is that by binding to the (dC) region mithramycin has caused an increase in the minor groove width of the neighbouring (dT) segment, rendering it more susceptible to DNAase I cleavage. Neither actinomycin nor echinomycin, which bind to the sequences GpC and CpG respectively, affect the cleavage in the CgT11 region of this fragment. In
Figure 5. DNAase I digestion of the pTyr 2 DNA. (a) Autoradiographs of the digestion pattern of the bottom strand in the presence of 2μM and 10μM mithramycin (MITH), olivomycin (OLIV) and chromomycin (CHRO). Time in minutes after the addition of enzyme is indicated at the top of each gel lane. The arrows indicate an error in loading order. (b) Differential cleavage plot for pTyr 2 DNA in the presence of 10μM mithramycin.

contrast distamycin causes an enhanced rate of cleavage in the (dC) runs while completely protecting the (dT) segment. Presumably this occurs because the antibiotic binds to the (dT) section and induces a reduction in the minor groove width of the neighbouring (dC) run. If this interpretation in terms of minor groove width is correct then we can envisage two mechanisms by which mithramycin could induce this change. Either the antibiotic binds to the minor groove of the (dC) run forcing it to a wider structure than normal, or it binds in the major groove, reducing its width, thereby causing an increase in the width of the opposing minor groove. Both these mechanisms increase the local minor groove width although it is not possible to distinguish between them on the basis of the results presented here.

Mode of binding

While the above results demonstrate that mithramycin does cause distinct changes in susceptibility to DNAase I cleavage it is noticeable that the protected regions are less impressive than those produced by other sequence selective DNA binding ligands [18,19]. In contrast the enhanced rates of cleavage are a similar magnitude to those previously observed. Two simple theories can be advanced to account for this discrepancy. Either mithramycin binds to the DNA minor groove in such a way that access of the enzyme to the
phosphate backbone is not sterically hindered or the antibiotic binds from the major groove thereby causing changes in the width of the opposing minor groove. We have attempted to answer this question by examining the reaction with dimethyl sulphate. Dimethyl sulphate methylates N7 of guanine, lying in the major groove, allowing subsequent cleavage of the DNA with piperidine. Any ligand which binds to guanine residues from the major groove may reduce the rate of this reaction whereas binding in the minor groove is less likely to have any appreciable effect. The results with tyr T DNA are presented in Figure 7, along with those for actinomycin, nogalamycin and spermine included for comparison. As predicted, actinomycin D causes no change in this G specific reaction since it binds to the dinucleotide GpC in the minor groove [18,24]. Similarly spermine, which is usually found in the DNA major groove [25], produces no changes. In contrast both nogalamycin and mithramycin affect the reaction with dimethyl sulphate. The results are most easily interpreted for nogalamycin, which binds to DNA by the mechanism of intercalation with bulky sugar residues in both major and minor grooves. The relative intensities of bands at positions 22, 46, 70, 118 and 133 are clearly reduced in the presence of this ligand while enhancements are evident at position 95, 97, 98, 99, 139 and 140. All of the regions of reduced reaction are found in sequences which are protected from DNAase I cleavage [26]. The broad enhancements between positions 95-100 lie in a region in which DNAase I cutting is largely unaffected by nogalamycin [26]. The increased sensitivity to dimethyl sulphate in this region is probably caused by nogalamycin binding at 89-93 where DNAase I cutting is protected, a region containing no guanine residues on the lower strand. The results with mithramycin are less clear cut. The reaction at positions 70, 99 and 100 is clearly reduced while bands at positions 73, 78, 97 and 98 are enhanced. These changes do indeed correspond to regions with
Figure 7. Dimethyl sulphate-piperidine markers for guanine on tyr T DNA prepared in the presence of various ligands. Lane 1, control; lane 2, 20μM mithramycin; lane 3, 10μM nogalamycin; lane 4, 15μM actinomycin D; lane 5, 30μM spermine.
altered sensitivity to DNAase I. However, all the bonds for which reaction with dimethyl sulphate is affected (both enhancements and blockages) are protected from DNAase I cleavage so that it is not possible to assess the precise structural or steric changes occurring. Clearly mithramycin can affect the access of dimethyl sulphate to N7 of guanine but it is not obvious whether this is due to direct steric hinderance or structural changes induced by binding to the opposing groove. In similar experiments with pTyr 2 DNA, to which mithramycin does not bind very well, no changes were observed in the sensitivity to dimethyl sulphate.

We have also performed experiments using DNAase II as a footprinting agent. This enzyme has very different structural requirements to DNAase I and appears to cut best in runs of purines in duplexes with wide minor grooves possibly recognizing a stacked single strand of limited exposure [27]. We observed little or no changes in the sensitivity of tyr T or pTyr 2 DNA to cleavage by this enzyme in the presence of mithramycin.

DISCUSSION
Sequence-selectivity

The antibiotic-induced patterns of protection from DNAase I cleavage correlate well between the four DNA fragments used in this work, and the results help to clarify the base sequences which form the preferred binding sites for mithramycin. As previously reported [14] all the sites contain at least two contiguous GC base-pairs. What then is the exact arrangement of the bases in the preferred binding sequence; is it CpC (or GpG), GpC or CpG? We can easily discount the possibility that the ligand only recognizes the sequence CpG or GpC since the cleavage patterns are very different from those in the presence of both echinomycin and actinomycin [18,19]. We are inclined to the view that, while the antibiotic can interact with all three of these dinucleotide steps, the preferred arrangement is GpG. This can be most easily discerned by examining the results with Xbal DNA. The step GpG is found at positions 41, 43, 50, 56, 57, 61, 76, 77, 81, 82, 84, 89, 94 and 97 and of these only that at position 89 is totally unaffected by mithramycin. While many of the CpG and GpC dinucleotide steps are found immediately adjacent to these sites those at positions 28, 69 and 70 are not blocked by the presence of the antibiotics. This suggestion is confirmed by inspecting the data with tyr T and pTyr 2 DNAs. None of the CpG or GpC steps in pTyr 2 is affected by the antibiotic while the GpG at positions 48 and 98 form weak sites of protection. With tyr T DNA the GpC and CpG steps at positions 35, 36, 58, 78,
79, 94, 95, and 117 are not protected by the antibiotic while cleavage at all the GpG steps, except those at positions 23 and 40, is blocked.

Although DNAase I is not the best probe for estimating the limiting site sizes of DNA-binding ligands our results are consistent with previous suggestions that mithramycin binds to a minimum of three base-pairs [14]. It is not possible to determine whether the antibiotic specifically recognizes any particular trinucleotide sequence containing the step GpG although we note that the ligands do not bind to positions 40 of tyr T and 89 of XbsI which are surrounded by A and T residues.

Differences between antibiotics

The footprints obtained with chromomycin and olivomycin are similar, but not identical, to those produced by mithramycin. Chromomycin and olivomycin, which possess the same five sugar residues and only differ by a methyl group on the aglycon moiety produce cleavage patterns which are virtually identical. In general chromomycin and olivomycin, at the same concentrations, interact with all the mithramycin binding sites yet seem to be capable of recognizing other sites as well, albeit less strongly. The sequences protected by these antibiotics extend over a longer region than mithramycin suggesting that while the sugar residues play little role in determining the absolute sequence selectivity they do affect the relative binding strengths and conformational restrictions of the antibiotics.

Since little is known about the three-dimensional structures of these ligands in solution or when bound to DNA, it is not possible to correlate these changes in binding with any particular antibiotic conformation. However, it is tempting to speculate that the sugar rings in these antibiotics align themselves with the DNA phosphates via hydrogen bonds between the sugar hydroxyl groups and the DNA phosphate backbone. Their sequence specificity would then result from interaction between the chromophore and guanine (or cytosine) bases, while the sugars affect the binding strength.

Mode of binding

It has previously been suggested, from experiments with synthetic DNA polymers, that mithramycin binds to the DNA minor groove. The results presented here do not conclusively prove which groove the antibiotic binds to, and we cannot rule out the possibility that this occurs from the major groove. The regions protected from DNAase I cleavage are not as well defined as those produced by other sequence-selective ligands on the same DNA fragment [18,19]. This cannot be explained simply by invoking differences in the kinetics of interaction with DNA since mithramycin dissociates from DNA at a very similar
rate to actinomycin D [12,28]. In contrast the regions of enhanced cleavage are clearly defined and more easily discerned. This may simply reflect the different mechanisms of binding, echinomycin and actinomycin intercalate into the DNA double helix, while this is thought to be unlikely for mithramycin [15]. However, this may indicate that the ligand is not directly affecting the access of the enzyme to the DNA minor groove. The poor protection is particularly striking with the 65-mer containing the sequence C_gT_jj. Whilst distamycin completely protects the (dT) run from cleavage, mithramycin only reduces cutting in the (dC) segment by 3-4 fold. This may again suggest that protection is achieved by some indirect effect, possibly ligand binding to the major groove.

**Structural changes upon binding**

The results presented here once again demonstrate that a common feature of sequence-selective DNA-binding ligands is the ability to induce structural change in regions surrounding their binding sites. These enhancements have previously been explained by suggesting that when it occurs in an AT-rich region, it is because the neighbouring ligand induces a local widening of the minor groove restoring it to a more normal 'B-DNA' like structure [18,19]. Enhancements in GC-rich regions are understood to result from a reduction in the local minor groove width [18]. The enhanced rates of DNAase I cleavage produced by mithramycin are found in AT-rich regions and are generally in similar positions on the tyr T fragment to those observed with actinomycin and echinomycin [18,19]. The large enhancement produced between positions 80-90 on tyr T DNA is peculiar to this antibiotic and confirms its different sequence selectivity and/or mode of binding. How do these changes correlate with the known effects of mithramycin on DNA structure? Waring [15] demonstrated that mithramycin does not significantly unwind covalently closed circular DNA, although a small change in sedimentation coefficient was produced by chromomycin. These small differences in unwinding angle may be partly responsible for the different footprinting patterns produced by the various ligands. If these antibiotics do not significantly unwind DNA, how do they produce changes in the local groove width? One possible explanation could be that the additional strain imposed on covalently closed circular DNA, used in the unwinding experiments, causes it to adopt a structure which is locally similar to that induced by mithramycin binding, so that the ligand causes no perturbation of the supercoiled DNA structure. Alternatively, it is possible that the small structural changes needed to increase the rate of cleavage by DNAase I cannot be detected by simple biophysical techniques and serve to
emphasize the usefulness of this enzyme as a probe for DNA structure. However, a more likely explanation is that mithramycin affects the local groove width without unwinding the DNA helix, by altering the relative positioning of the two DNA strands, changing the cross-strand phosphate-phosphate distance. The enhancements produced by mithramycin are all restricted to AT-rich regions surrounding the ligand binding site and suggest that the antibiotic increases the local minor groove width. It remains to be confirmed whether this is a direct consequence of drug binding to the minor groove or is produced via the drug interacting with the major groove.

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REFERENCES