The GC-selective ligand mithramycin alters the structure of $(AT)_n$ sequences flanking its binding sites

Benjamin M.G. Cons and Keith R. Fox

Dept. Physiology & Pharmacology, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, UK

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DNA fragments containing $(AT)_n$ inserts cloned adjacent to putative mithramycin binding sites have been examined by footprinting experiments using a variety of nucleases in the presence of the drug. The results demonstrate that mithramycin induces a DNA structural change which renders adjacent $(AT)_n$ sequences sensitive to attack by DNase II. Significant changes are also revealed with DNase I and micrococcal nuclease. The results are consistent with a model in which mithramycin opens the DNA minor groove changing it to a structure which is locally more like A-DNA.

Mithramycin; DNase I; Footprinting; DNA structure; $(AT)_n$

1. INTRODUCTION

Mithramycin is an antitumour antibiotic which acts by binding to DNA thereby inhibiting its function as a template for DNA-directed RNA polymerase [1,2]. This interaction requires magnesium [3] and the presence of guanine bases [4]. Previous footprinting studies using methidiumpropyl-EDTA [5], DNase I [6], and hydroxyl radicals [7] have confirmed this selectivity and suggested that the recognition site consists of the dinucleotide step GpG.

Since nucleases produce DNA cleavage patterns which are dependent on local DNA structure they can be used to report conformational changes which accompany drug binding. DNase I footprinting studies have revealed that many ligands cause increases in enzyme activity in regions surrounding their binding sites [8,9]. Previous footprinting studies have either used natural DNAs of random sequence [5–9] or short oligonucleotides [10,11] as substrates. Enhancements in the former are difficult to interpret rigorously because of the large number of closely related binding sites, while the latter can be criticised on account of the short size of the DNA which may be subject to end effects. In this paper we investigate the effects of mithramycin on nuclease digestion patterns of DNA fragments containing regions of alternating A and T adjacent to isolated mithramycin binding sites.

Correspondence address: K.R. Fox, Dept. Physiology and Pharmacology, University of Southampton, Bassett Crescent East, Southampton SO9 3TU, UK

2. MATERIALS AND METHODS

2.1. *Drugs and enzymes*

Mithramycin was a gift from Pfizer USA and was stored as a 2 mM stock solution in 10 mM Tris-HCl containing 10 mM NaCl. DNase I, DNase II and micrococcal nuclease were purchased from Sigma.

2.2. *DNA fragments*

Plasmids containing alternating AT inserts cloned within the *Sma*I site (CCC/GGG) were prepared as follows. Poly(dA-dT) (Boehringer) was cut to short lengths by mild digestion with DNase I and the fragments were blunt-ended by filling with dATP and dTTP using reverse transcriptase. These were then ligated into *Sma*I cut pUC13 and transformed into *E. coli* TG2. Transformants were picked in the usual way as white colonies and their sequences were determined by Maxam-Gilbert reactions. The 3 fragments used in this work contained $(TA)_5$, $(AT)_6A$ and $(TA)_{10}$ in the *Sma*I site within the local sequence TCGCCC/GGGGAT. Radiolabelled DNA fragments containing these inserts were prepared by digesting the plasmids with *Hin*dIII, labelling with [α-^{32}P]dATP and cutting again with *Eco*RI. These were then purified from 8% polyacrylamide gels.

2.3. *Footprinting and gel electrophoresis*

Footprinting with DNase I, DNase II and micrococcal nuclease (MNase) was performed as previously described [8,9,12]. In each case 1 mM $MgCl_2$ was included in the reaction since this is required for mithramycin binding [4]. The products were resolved on 10% (w/v) polyacrylamide gels containing 8 M urea. Bands in DNase I digests were assigned by comparison with Maxam-Gilbert sequencing reactions. Since DNase II and MNase generate radiolabelled products which terminate in a 5′-hydroxyl, in contrast to DNase I and Maxam-Gilbert reactions which end in a 5′-phosphate the products of the two reactions have different gel mobilities. Products from MNase cleavage were easily assigned since this enzyme cuts only pA and pT bonds [12], these were then used as markers for DNase II digestion.

3. RESULTS

3.1. *DNase I footprinting*

Fig. 1 presents DNase I digests of the $(TA)_{10}$, $(TA)_6T$ and $(TA)_5$ containing DNA fragments in the presence

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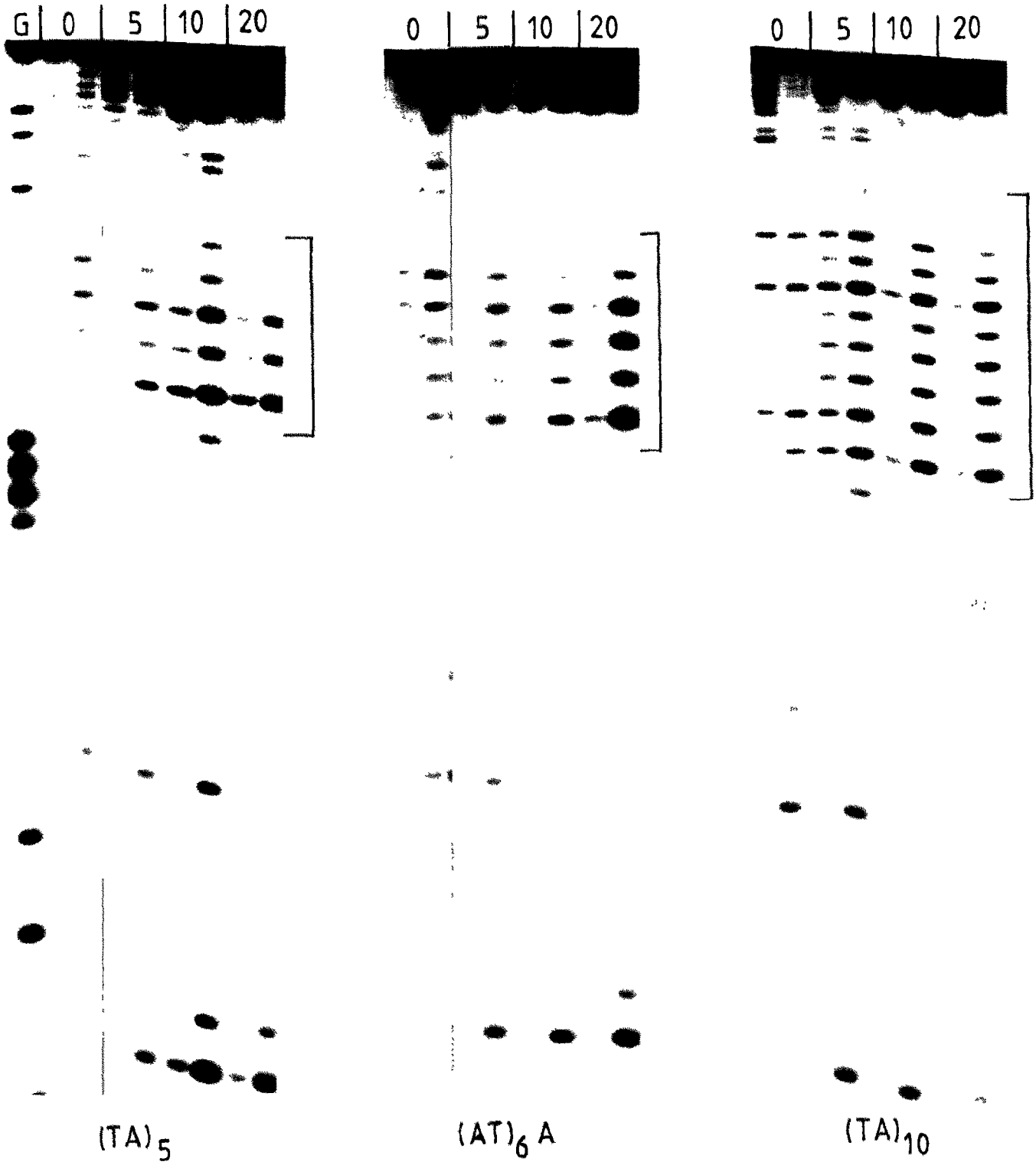

Fig. 1. DNase I digests of the $(TA)_5$, $(AT)_6A$ and $(TA)_{10}$ containing DNA fragments in the presence and absence of mithramycin. Each fragment labelled at the 3'-end of the *Hin*dIII site. The square brackets indicate the position of the inserts. The track labelled G is a Maxam-Gilbert dimethylsulphate piperidine marker specific for guanine. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. Drug concentrations (μM) are shown at the top of each pair of lanes.

and absence of various concentrations of mithramycin. In the absence of the antibiotic all 3 fragments show cleavage at TpA but not ApT as expected [13,14]. In the presence of mithramycin clear footprints can be seen at either end of the inserts corresponding to the sequence GGG (CCC). More bonds are protected

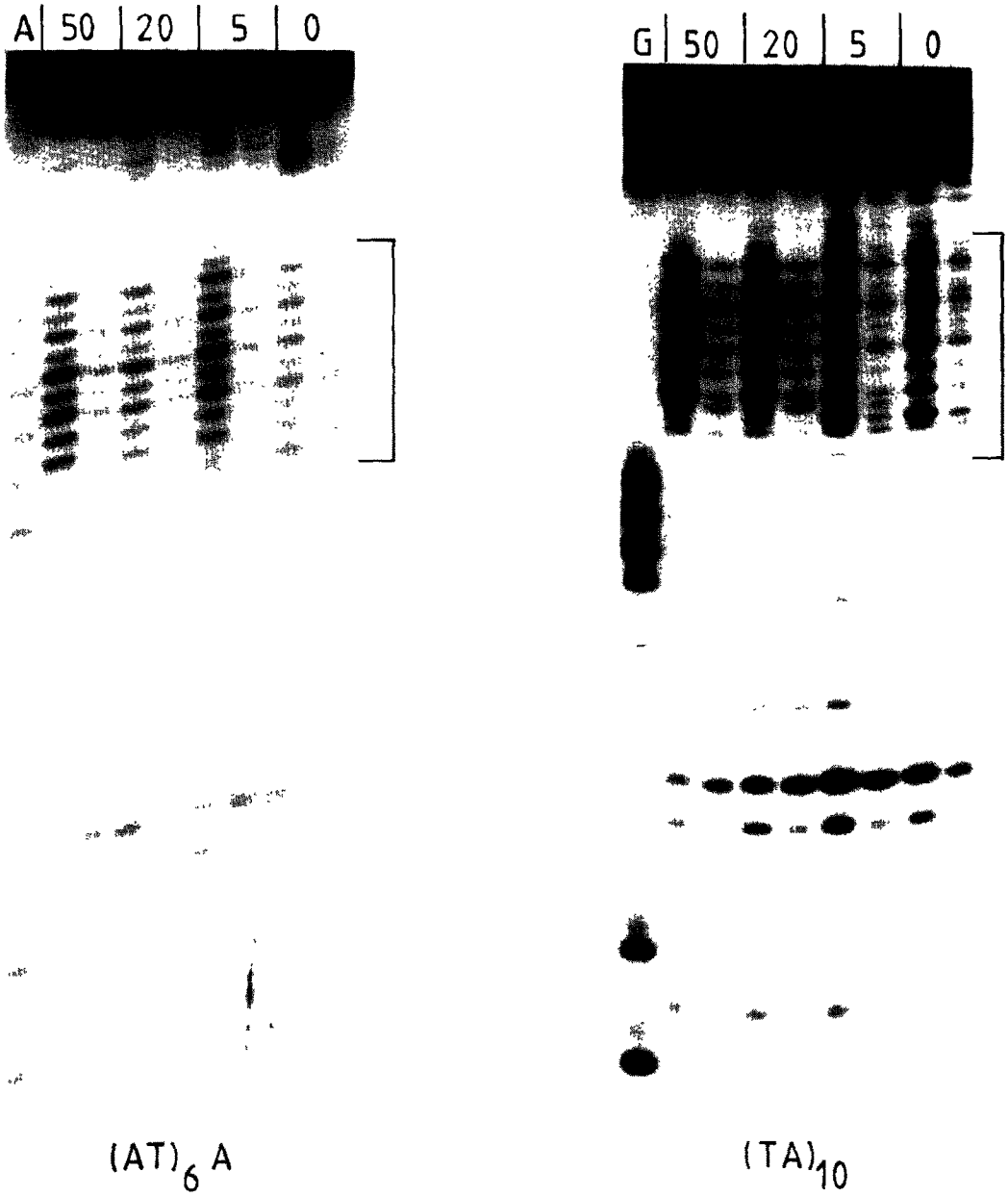

Fig. 2. MNase digests of the $(AT)_6A$ and $(TA)_{10}$ containing DNA fragments in the presence and absence of mithramycin. The square brackets indicate the position of the inserts. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min (reading right to left). The track labelled 'A' corresponds to a K_2PdCl_4-piperidine marker specific for adenine [22]. Drug concentrations (μM) are shown at the top of each pair of lanes.

towards the upper (5′) side of the insert, since DNase I footprints are staggered towards the 3′-side of drug binding sites. In the presence of mithramycin, the preference for cutting ApT over TpA is retained and cleavage products from the latter are still barely visible. However, in each case there appears to be a significant increase in cutting throughout the insert. In the absence of quantitative footprinting studies it is not possible to assess whether this reflects a change in DNA conformation or if it is simply due to alterations in the relative concentrations of free DNA and enzyme.

3.2. *Micrococcal nuclease (MNase)*

Fig. 2 presents the results of similar studies using MNase as the footprinting probe. This enzyme cuts exclusively at pA and pT bonds and is particularly active in regions of alternating AT [13,14]. Cleavage is slightly better at TpA than ApT. In the presence of mithramycin, the outermost MNase cleavage products are missing, presumably because of some steric occlusion by the adjacent drug molecule. In each case cleavage within the insert is increased and the cutting pattern is more even. This is especially noteworthy

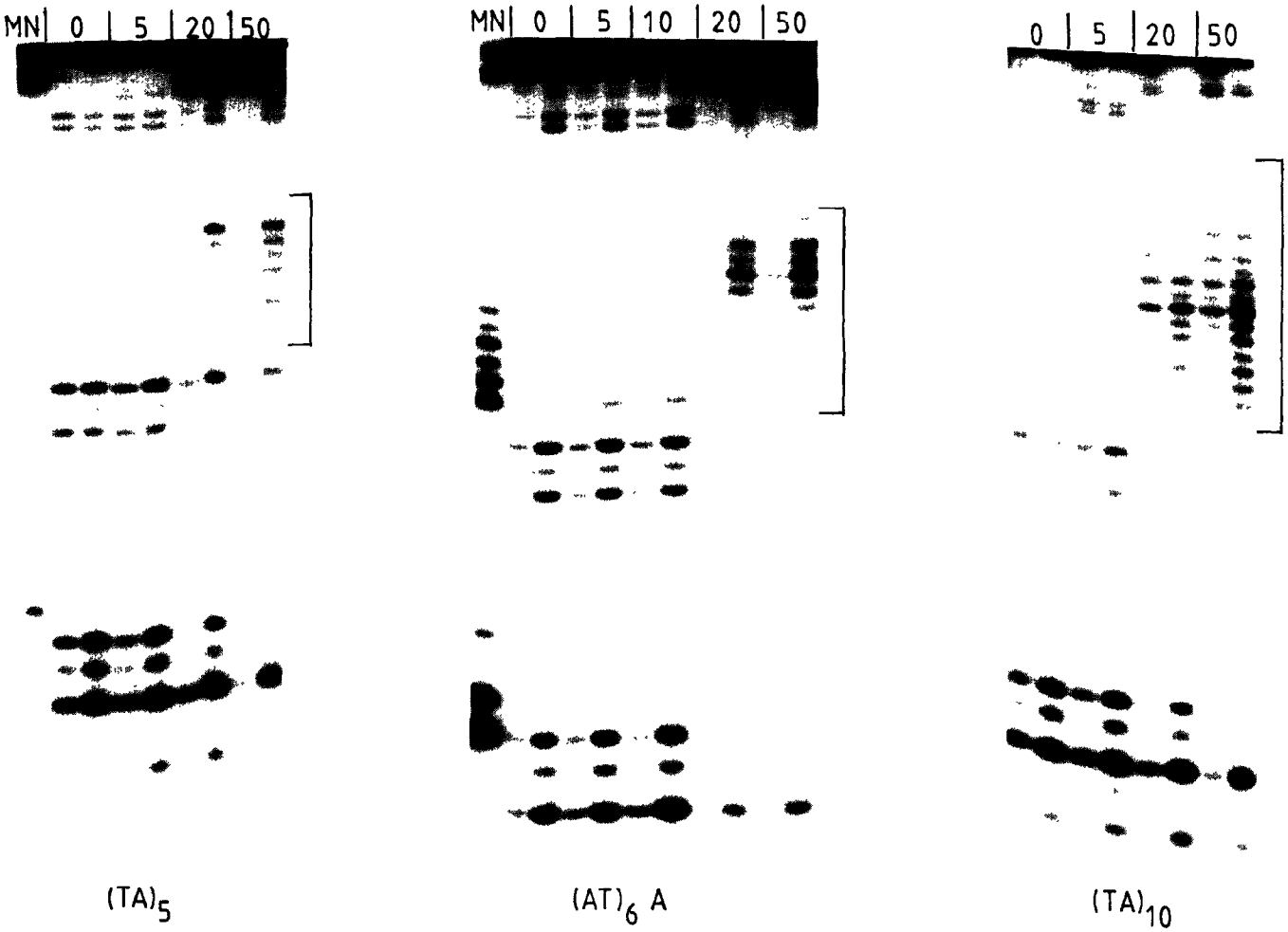

Fig. 3. DNase II digests of the $(TA)_5$, $(AT)_6A$ and $(TA)_{10}$ containing DNA fragments in the presence and absence of mithramycin. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 min. The tracks labelled MN correspond to the products of MNase digestion and are useful for assigning the pA and pT bonds (see Fig. 2). The square brackets indicate the positions of the inserts.

since MNase attack at other bonds outside the insert does not appear to have been altered.

3.3. *DNase II*

Fig. 3 presents the results of DNase II digestion of the same 3 DNA fragments in the presence and absence of mithramycin. This enzyme cuts the 05′–P bond [15,16] especially in runs of purines, so that cleavage does not correlate across the two strands. It appears to recognise a peculiar DNA strand conformation which is possibly close to an A-like structure. As previously noted, this enzyme shows no cleavage in AT inserts [13]. In the presence of mithramycin, cutting is greatly reduced around the GGG (protection at the upper CCC is not apparent since this is a very poor DNase II site). More interesting, however, are the novel cleavage products apparent on the drug-treated lanes. It appears that mithramycin has somehow rendered portions of the AT inserts sensitive to DNase II attack. In each case the increased cleavage is greatest 8 bases from the 3′-end of the insert.

4. DISCUSSION

4.1. *Structural changes induced by mithramycin*

Mithramycin binds in a non-intercalative fashion in the DNA minor groove. NMR studies have suggested that the drug binds as a dimer, coordinating a single magnesium ion, and that this large species causes a dramatic widening in the local width of the minor groove [17,18]. Does this binding model explain the observed changes in enzyme activity? The most dramatic changes are seen with DNase II. Although the precise structural requirements of this enzyme are not certain, it is known to cut the DNA strands asymmetrically with the best cleavage in runs of purines (especially G or G and A) [15,16]. It has been proposed that the enzyme binds to one strand of the duplex and is sensitive to the orientation of 3 adjacent phosphate groups [15]. It may be that this enzyme cuts DNA best when the strand conformation resembles something akin to A-DNA, with a very wide and shallow minor groove. Such a conformation has been proposed at the

mithramycin binding site [17,18]. It therefore seems that this altered conformation is transmitted into the adjacent runs of A and T.

There is only one report of DNase II footprinting of mithramycin on a natural DNA fragment [19]. In this case, although drug-induced protections were observed, no enhancement of enzyme activity was evident. However, this may be because this DNA sequence did not contain any regions of alternating AT; these may be peculiarly sensitive to drug-induced changes in structure.

Although DNase I cleavage at ApT is increased, cutting of TpA appears to be unaffected, so that the alternating pattern of products is still apparent. The simplest interpretation of the DNase I data would be that mithramycin has further stabilised the alternating B-structure. Previous DNase I footprinting studies with tyrT DNA revealed enhancements in enzyme activity in the sequence ATAT (position 88) [6].

The changes in cleavage pattern observed with these DNA fragments are similar for all the different length inserts. The structural changes occur in an all-or-none fashion, consistent with the cooperative binding that has been detected for binding of several ligands to poly(dA-dT).

4.2. *Comparison with other drugs*

Actinomycin [20] and echinomycin [21] also show changes in enzyme activity in regions of alternating AT surrounding their binding sites. How do these results with mithramycin compare with other data? Actinomycin and echinomycin each alter the relative rates of DNase I cleavage at TpA and ApT so that the preference for cutting ApT is less pronounced. This contrasts with mithramycin which leaves the TpA step in an unreactive conformation. The changes in reactivity of MNase are similar for all 3 drugs, causing an increased rate of cleavage in which both TpA and ApT bonds are cut at equal rates. Actinomycin induces DNase II cleavage in regions of alternating A and T which are sandwiched between two GpC sites; these effects increase towards the centre of the insert in much the same way as mithramycin.

Two important points can be drawn from these observations. Firstly the changes in enzyme activity are different for various ligands. Each drug induces characteristic changes in DNA structure. Secondly the results emphasise the importance of using a wide range of enzymic probes for studying the effects of drugs on DNA structure.

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REFERENCES

[1] Kersten, W., Kersten, H. and Szybalski, W. (1966) Biochemistry 5, 236–244.
[2] Gause, G.F. (1975) in: Antibiotics III, Mechanism of Action of Antimicrobial and Antitumour Agents (Corcoran, J.W. and Hahn, F.E. eds) pp. 197–202, Springer, Berlin.
[3] Cons, B.M.G. and Fox, K.R. (1989) Biochem. Biophys. Res. Commun. 160, 517–524.
[4] Behr, W., Honikel, K. and Hartmann, G. (1969) Eur. J. Biochem. 9, 82–92.
[5] Van Dyke, M.W. and Dervan, P.B. (1983) Biochemistry 22, 2373–2377.
[6] Fox, K.R. and Howarth, N.R. (1985) Nucleic Acids Res. 13, 8695–8714.
[7] Cons, B.M.G. and Fox, K.R. (1989) Nucleic Acids Res. 17, 5447–5459.
[8] Low, C.M.L., Drew, H.R. and Waring, M.J. (1984) Nucleic Acids Res. 12, 4865–4879.
[9] Fox, K.R. and Waring, M.J. (1984) Nucleic Acids Res. 12, 9271–9285.
[10] Lane, M.J., Laplante, S., Rhefuss, R.P., Borer, P.N. and Cantor, C.R. (1987) Nucleic Acids Res. 15, 839–852.
[11] Huang, Y.-Q., Rehfuss, R.P., LaPlante, S.R., Boudreau, E., Borer, P.N. and Lane, M.J. (1988) Nucleic Acids Res. 16, 11125–11139.
[12] Fox, K.R. and Waring, M.J. (1987) Biochim. Biophys. Acta 909, 145–155.
[13] Suggs, J.W. and Wagner, R.W. (1986) Nucleic Acids Res. 14, 3703–3716.
[14] McClellan, J.A., Palecek, E. and Lilley, D. (1986) Nucleic Acids Res. 14, 9291–9309.
[15] Drew, H.R. (1984) J. Mol. Biol. 176, 535–557.
[16] Drew, H.R. and Travers, A.A. (1984) Cell 37, 491–502.
[17] Gao, X. and Patel, D.J. (1989) Biochemistry 28, 751–762.
[18] Gao, X. and Patel, D.J. (1989) Q. Rev. Biophys. 22, 93–138.
[19] Cons, B.M.G. and Fox, K.R. (1990) Anti-Cancer Drug Design 5, in press.
[20] Waterloh, K. and Fox, K.R. (1990) Anti-Cancer Drug Design 5, in press.
[21] Fox, K.R. and Kentebe, E. (1990) unpublished observations.
[22] Iverson, B.L. and Dervan, P.B. (1987) Nucleic Acids Res. 15, 7823–7830.