High resolution hydroxyl radical footprinting of the binding of mithramycin and related antibiotics to DNA

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ABSTRACT

The preferred binding sites for mithramycin on three different DNA fragments have been determined by hydroxyl radical footprinting. Sequences which appear as one long protected region using DNAase I as a footprinting probe are resolved into several discrete binding domains. Each drug molecule protects three bases from radical attack, though adjacent regions show attenuated cleavage. Mithramycin and the other related compounds induce similar footprinting patterns and appear to recognise GC rich regions with a preference for those containing the dinucleotide step GpG. The ability of each such site to bind the drug depends on the sequence environment in which it is located. The data are consistent with mithramycin binding to the DNA minor groove.

INTRODUCTION

Mithramycin, chromomycin and olivomycin (Figure 1) are closely related antitumour antibiotics of the aureolic acid group [1−3]. Their antitumour activity has been attributed to the ability to bind double-stranded DNA, thereby blocking its function as a template for DNA-directed RNA polymerase [4−7]. This interaction with DNA requires divalent metal ions [8,9] (especially magnesium) and the presence of guanine bases [8,10]. Previous footprinting studies using methidiumpropyl-EDTA (MPE) [11] and DNAase I [12] have confirmed this broad selectivity and suggested that the drug specifically recognises the dinucleotide step GpG (CpC).

Although DNAase I is a widely used and highly successful footprinting probe it has several drawbacks. Because of its bulk it is not useful for rigorously determining the size of ligand binding sites and in previous studies with mithramycin large footprints were observed, corresponding to several overlapping sites which could not be resolved. A second problem encountered when using DNAase I is that its cleavage pattern is not even, but depends on local DNA structure. As a result ligands which bind to regions in which enzyme cutting is poor present badly defined footprints. Drugs which modify DNA structure in regions surrounding their binding sites can also induce enhanced rates of enzyme cleavage which further complicates the interpretation of footprinting patterns. An approach which circumvents these problems is the use of hydroxyl radicals as footprinting tools [13−15]. These produce an even ladder of cleavage, which is only weakly dependent on DNA sequence and, because of their very small size, are able to map out binding sites with very high precision. As with DNAase I footprinting the method was first applied to the binding of proteins to DNA [14]. Previous attempts to use hydroxyl radicals for probing small molecule-DNA interactions have met with limited success. Although distamycin and related compounds produce clear hydroxyl radical footprints [16], actinomycin and nogalamycin do not [17].

In this paper we describe the use of hydroxyl radicals to map the binding sites for mithramycin on three different DNA fragments, and compare these to patterns obtained with some novel naturally occurring derivatives as well as with DNAase I footprints. The results are discussed in the light of a recent NMR study [18,19] which suggests that mithramycin binds as a dimeric molecule within the DNA minor groove.

MATERIALS AND METHODS.

Drugs and enzymes. Mithramycin was a gift from Pfizer Inc. U.S.A. and was prepared as a 1mM solution in 10mM Tris-HCl pH 8.0 and stored in the dark at 4°C. Demethylchromomycin A2 and A3 and demethylolivomycin A and B [20,21] were obtained from Dr. M. Koenuma, Shionogi Research Labs. Osaka, Japan. Chromomycin A3 was purchased from Sigma. Because of their lower solubilities solutions of these analogues were prepared by dissolving small amounts in 10mM Tris-HCl pH 8.0 containing 10mM NaCl, separating any undissolved drugs by centrifugation, and estimating the concentration on the basis of the OD_{400} by comparison with a known solution of mithramycin. DNAase I was purchased from Sigma and stored as a 7200 units/ml solution at −20°C.

DNA fragments. TyrT DNA was prepared and 3′-end labelled with either α-^{32}P-dATP (EcoR1 end) or α-^{32}P-dCTP (AvaI end) using reverse transcriptase as previously described [12,17]. The 135 and 220 base pair DNA fragments were prepared as previously described [17] from plasmid pXbs1 [22] by cutting with HindIII, labelling the 3′-end with α-^{32}P-dATP and reverse transcriptase and cutting again with Sau3A1. The radiolabelled fragments were separated on a 6% (w/v) polyacrylamide gel.

Footprinting. The DNA concentration in all footprinting experiments was about 1μM base pairs (typically 5 − 10nM strand concentration), so that in each case there is a molar excess of drug molecules. As a result the binding ratio of drug/DNA is limited by the equilibrium binding constant of the reaction rather than by their stoichiometric ratios. DNAase I footprints were performed as previously described [12,17]. Hydroxyl radical footprinting was performed by mixing 10μl of a drug-DNA complex with 10μl of a freshly prepared mixture containing 0.1mM EDTA, 2.5mM ascorbic acid, 0.07% (v/v) hydrogen peroxide

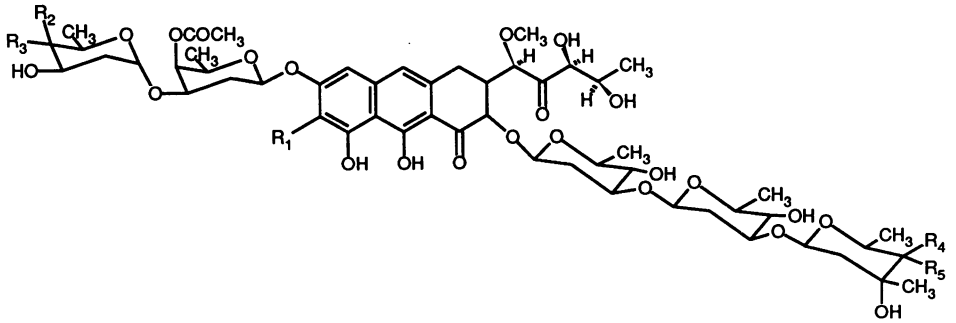


Figure 1. The structure of mithramycin and related analogues.
Mithramycin: R_1=CH_3, R_2=R_4=H, R_3=R_5=OH
Demethylchromomycin A_3: R_1=CH_3, R_2=OH, R_3=R_5=H, R_4=$COCH_3$.
Demethylchromomycin A_2: R_1=CH_3, R_2=OH, R_3=R_5=H, R_4=$COCH(CH_3)_2$.
Chromomycin A_3: R_1=CH_3, R_2=OCH_3, R_3=R_5=H, R_4=$COCH_3$.
Demethylolivomycin A: R_1=R_3=R_5=H, R_2=OH, R_4=$COCH(CH_3)_2$.
Demethylolivomycin B: R_1=R_3=R_5=H, R_2=OH, R_4=$COCH_3$.

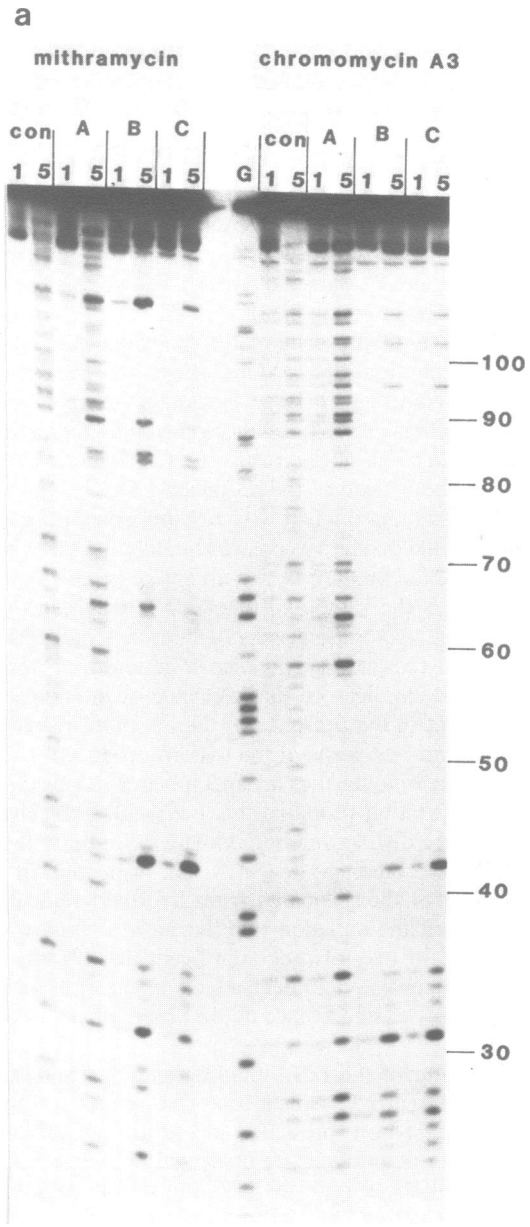
and 50μM ferrous ammonium sulphate. The reaction was stopped after 3mins by adding 10μl of 100mM thiourea, followed by lyophilisation. The sample was redissolved in formamide containing 10mM EDTA and 0,1% bromophenol blue. The products of the footprinting reactions were separated on 8% (w/v) polyacrylamide gels containing 8M urea. After electrophoresis at 1500 volts for about 2 hours the gel was fixed in 10% acetic acid, transferred to Whatman 3MM paper, dried under vacuum at 80°C and subjected to autoradiography at −70°C with an intensifying screen. Bands in the digests were assigned by comparison with dimethylsulphate- piperidine marker lanes specific for guanine.
Densitometry. Autoradiographs of the products of hydroxyl radical cleavage were scanned with a Joyce-Loebl Chromoscan 3 microdensitometer.

RESULTS.

DNAase I footprinting. Autoradiographs of DNAase I digestion patterns of the 135 base pair DNA fragment in the presence and absence of mithramycin, chromomycin and related analogues are presented in Figure 2. We have recently shown that the interaction between mithramycin and magnesium is not strong [9] ($K=10^3M^{-1}$), as a result all the footprinting experiments were performed in the presence of 10mM magnesium chloride, unless stated otherwise. In each case concentrations of 50μM and above produce digestion patterns which are very different from the control with large blockages between positions 44−58 and 71−83. Both of these sites contain GC rich regions so that they represent the sum of several overlapping binding sites which can not be resolved. The clearest instance of an isolated site is between 36−41, on the 3'-side of the sequence GGCC. On the basis of this data alone it is evident that mithramycin binds to GC rich regions with a site size of no more than 6 base pairs, and that all the derivatives possess broadly the same sequence recognition properties. In order to define the binding sites with greater precision we have performed hydroxyl radical footprints on the same and other DNA fragments.
Hydroxyl radical footprinting. Autoradiographs of hydroxyl radical cleavage of the 135 base pair DNA fragment in the presence and absence of mithramycin and the other analogues are presented on Figure 3. It can be seen that cleavage of the control produces a fairly even ladder of bands and that in the presence of the antibiotics several sites of reduced cleavage are evident. Densitometer scans of the pattern produced by 50μM mithramycin are presented in Figure 5. The binding sites are much better resolved than those produced using DNAase I as the footprinting probe so that the single protection between 44−58 is evidently composed of three distinct binding domains. Similarly the blockage between 71−83 consists of at least two binding sites. A visual inspection of the autoradiograph suggests that the drug protects about 3 bases from hydroxyl radical attack, though the densitometer scans reveal that the situation may be more complex with up to 5 bands showing attenuated cleavage. It may also be significant that certain bands appear to be slightly more susceptible to hydroxyl radical attack in the presence of the antibiotics especially 62−64 and 88−90. The significance of this observation will be considered further in the Discussion.

In order to define the nature of the DNA binding sites for mithramycin with greater certainty we have performed hydroxyl radical footprinting on two other DNA fragments and the autoradiographs obtained are presented in Figure 4. Densitometer scans of the lanes corresponding to 50μM mithramycin are presented in Figure 5. DNAase I footprints for tyrT DNA have previously been reported [12] and reveal large blockages in the GC rich regions between 70−80 and 95−120. Hydroxyl radical footprints of this DNA fragment

labelled on either strand show that these regions consist of several closely spaced discrete binding sites. The protected regions for all the fragments investigated are shown in Figure 6. The data for TyrT DNA show clearly that the protected regions are staggered across the two DNA strands in the 3′ direction, consistent with the hydroxyl radical probe attacking



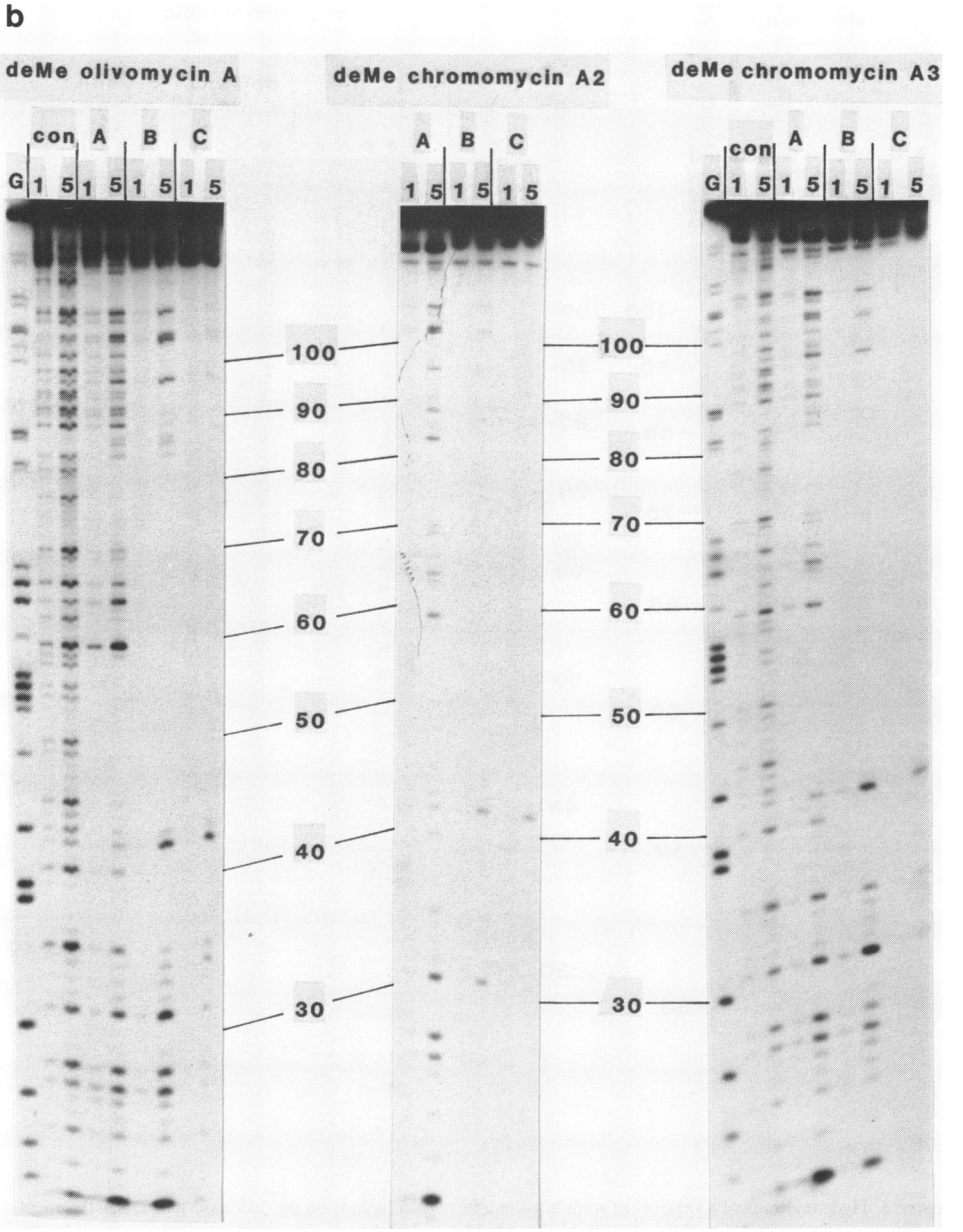


Figure 2. DNAase I footprinting of mithramycin and related analogues on the 135 base pair DNA fragment. a) Chromomycin A3 and mithramycin, b) demethyl derivatives of olivomycin A chromomycin A2 and chromomycin A3. the concentrations of the ligands were 0μM (con), 10μM (A), 50μM (B), 250μM (C). Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 mins. The tracks labelled 'G' are Maxam-Gilbert markers specific for guanine. The sequence numbers correspond to those shown in Figure 6.

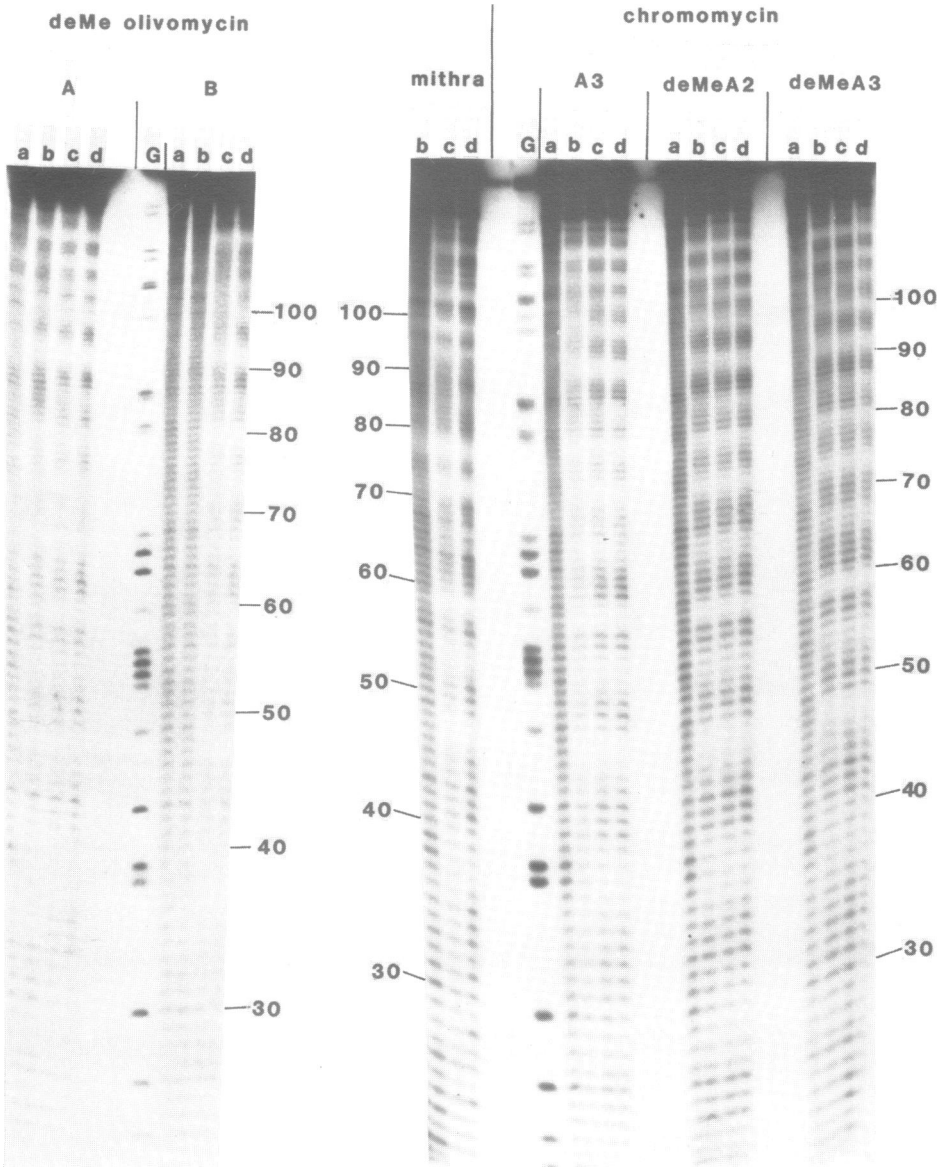


Figure 3. Hydroxyl radical footprints of mithramycin and related ligands on the 135 base pair DNA fragment. The drug concentrations were 0μM (a), 10μM (b), 50μM (c), 250μM (d). The track labelled 'G' is a Maxam-Gilbert marker lane specific for guanine.

the DNA from the minor groove. Where data for the two strands overlap it is clear that each of the blockage sites is centred around a GC rich region. These can be seen at the trinucleotide steps GCG(74), CGC(77), CGC(96), CGC(100) and CCG(106). In the regions

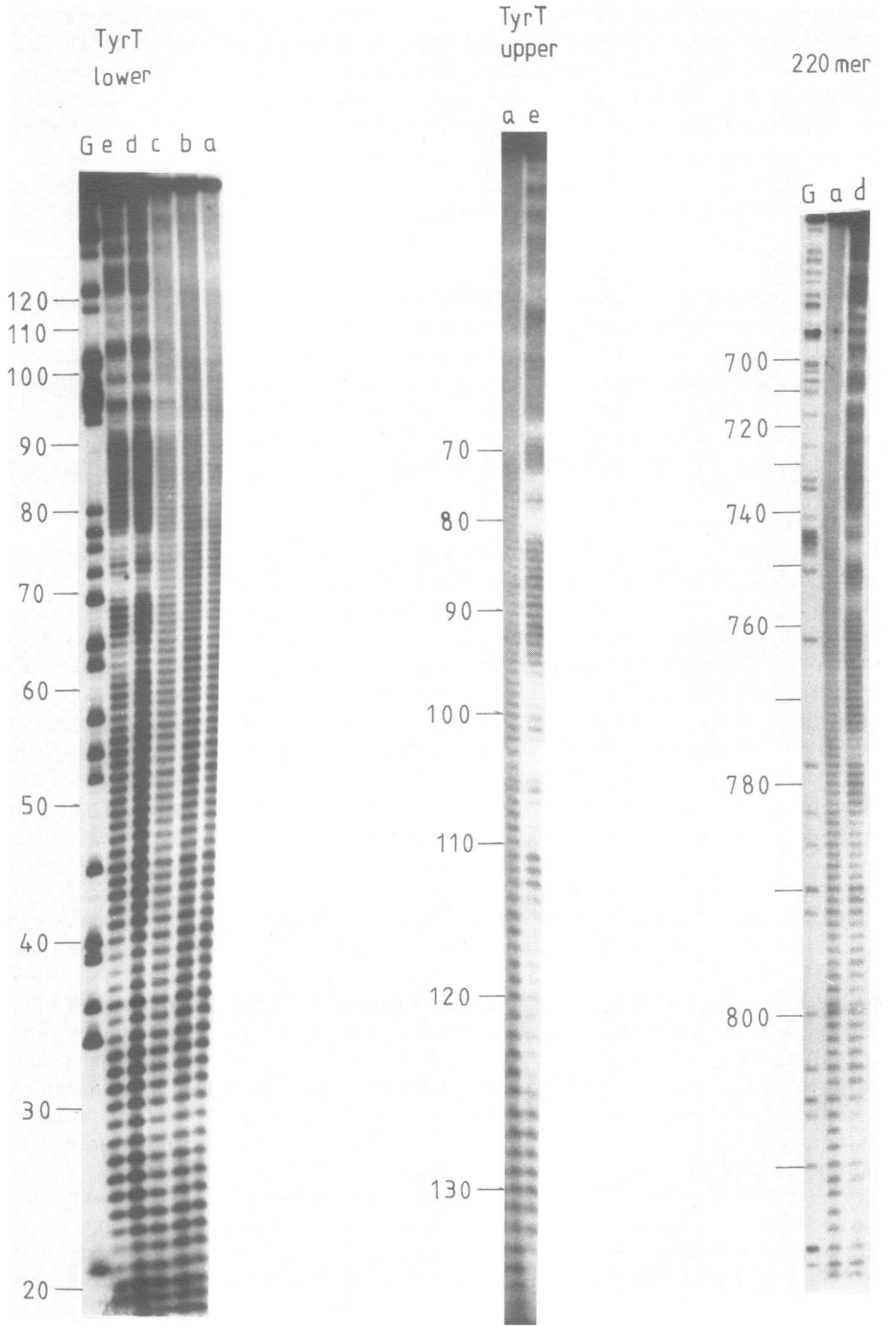
between 94−102 (GCGCCCCGC) and 71−77 (GCGGCGC) the protection is not even; each GC-rich domain contains a central portion which is less well protected. This may be related to the ability of mithramycin to discriminate between GC-containing sequences and will be considered further in the discussion. An unusual binding site is evident around position 65 in the sequence ACAC, visible on both DNA strands. The 220mer also reveals several discrete binding sites which are located to the 3′-side of GC rich regions. Generally these are associated with the dinucleotide step CpC (GpG) though not all such steps are protected; witness the lack of any inhibition around position 800(CCTGCCAG).

DISCUSSION

The data presented show that hydroxyl radicals can be used as a probe for investigating the binding of mithramycin to DNA. Clear footprints are obtained in which each binding site consists of three well-protected bases and up to three bases which show an attenuated cleavage pattern. Where a direct comparison with DNAase I footprints is possible the data for hydroxyl radicals yield much clearer footprinting patterns. Regions which contain several closely spaced binding sites appear as a single long protection with DNAase I yet are resolved into several discrete binding domains using hydroxyl radicals. The results reveal several details concerning the interaction of mithramycin with DNA which are best discussed under separate headings.

Mode of binding. The fact that mithramycin produces a hydroxyl radical footprinting pattern is significant in itself. Although the exact chemistry of this cleavage reaction has not been reported it is clear that they act by free radical attack on the deoxyribose sugars, and that this probably occurs from the DNA minor groove (witness the 3′-stagger in the cleavage pattern of TyrT DNA). Several other ligands, such as actinomycin and nogalamycin which are known to bind to DNA with substituents located in the minor groove fail to produce clear hydroxyl radical footprints [17]. The only other example of a small ligand successfully footprinted with this free radical probe is distamycin [16] which again is known to bind in the minor groove. The location of mithramycin on DNA has been a matter of considerable debate. On the basis of a previous footprinting study using dimethylsulphate and methylene blue as chemical probes as well as DNAase I, it was suggested that the drug *might* bind via the DNA major groove [12], though the evidence was equivocal, and the magnesium concentration may not have been sufficient. Using a wide variety of chemical probes reacting in the DNA major groove we have failed to detect mithramycin binding (Cons & Fox, unpublished observations). NMR studies have located the ligand within both the DNA major [23,24] and minor [18,19] grooves. We are of the opinion that the present footprinting data are best explained by a model in which mithramycin is bound to the DNA minor groove. A recent NMR study has suggested that chromomycin binds as a dimer within the DNA minor groove, and in so doing fills this groove, widening it, so that it more nearly resembles an A-like DNA structure. Although the data presented in this paper can not confirm this model it does account for the clear footprints observed, which suggest that the ligand is occluding a large portion of the DNA surface in the minor groove and severely restricting access to the sugar residues.

Sequence selectivity. The antibiotic-induced patterns of protection from hydroxyl radical cleavage are very similar for all the derivatives of mithramycin, olivomycin and chromomycin studied in this paper. Protections from hydroxyl radical attack occur in the same positions for all the ligands (Figure 3). This in itself is not surprising since the only differences between the compounds are in methyl groups not likely to be directly involved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in the sequence recognition process, but may modify the strength of the interaction. A previous study using MPE-Fe(II) as the footprinting probe also showed nearly identical patterns for mithramycin, chromomycin and olivomycin [11]. In contrast to DNAase I and MPE-Fe(II) footprints [11,12], which vary according to the ligand concentration, the footprinting patterns obtained with hydroxyl radicals appear to be all-or-none. Densitometer scans of hydroxyl radical footprints at low drug concentrations reveal that all the protected regions appear together within the range of concentrations examined; no sites are preferentially affected. This may reflect the ability of DNAase I to detect weakly bound ligands which may be associated with secondary binding sites. For any one compound all the hydroxyl radical footprints appear at similar ligand concentrations, so that within these tight binding sites there does not appear to be any strong binding selectivity. It is worth noting that the footprinting patterns with the chromomycin and olivomycin derivatives appear at lower concentrations than those for mithramycin, suggesting a tighter binding to DNA, as previously noted [12], and consistent with their lower aqueous solubilities.

These results are broadly similar to those obtained with MPE-Fe(II) which showed that the drugs had a minimum requirement of two contiguous base pairs and a minimum binding site size of three base pairs [11]. The similarity extends to the observation that both probes reveal that identical trinucleotides in nonidentical locations have different affinities. The data with MPE-Fe(II) report a protected region around a trinucleotide (GTG) similar to that observed at position 65 of TyrT DNA, reported in this paper. However, MPE-Fe(II) footprinting suggested that the various drug binding sites were not identical and could be ordered according to the drug concentration at which protection first appeared, whereas hydroxyl radicals produced an all-or-none pattern. This difference must reflect the nature of the probe. In this regard it is worth noting that MPE-Fe(II) cleavage requires a prior distortion of DNA structure by virtue of the intercalation of the methidium chromophore, whereas hydroxyl radicals are presumably more passive cleavage agents.

Almost all the ligand binding sites cluster around regions of high GC content. What then is the precise sequence selectivity? If we consider those sequences which are composed of only GC residues then there are three possible dinucleotides (GG=CC, GC, CG) and four trinucleotides (GGG=CCC, GGC=GCC, GCG=CGC, CGG=CCG). A dinucleotide alone does not appear to provide sufficient sequence information since several such isolated steps are unaffected for example 135mer position 96 (GG), tyrT 58 (CG), 220mer 762 (GC). Although it could be argued that these dinucleotide steps are not good binding sites by virtue of the nature of the surrounding sequences, the simplest explanation is that some other information is required to define the mithramycin recognition site. However, two contiguous GC base pairs do occasionally provide a good mithramycin binding site for example TyrT position 40 (GG) 220mer position 741 (GC) and 756 (CC). When trinucleotides consisting of GC residues are considered then 135-mer position 68 (GCG) and 220-mer position 801 (GCC) are representative ones which are not protected. By comparison the sequence TGCC is protected at position 787 on the 220mer but not at position 800. The factors affecting the ability of mithramycin to recognise a particular sequence appear to be complex and may indeed be affected by the structural environment

Figure 4. Hydroxyl radical footprinting of mithramycin on the TyrT and 220 base pair DNA fragments. For the TyrT DNA fragment 'upper' and 'lower' refer to the different labelled strands as shown in Figure 6. The drug concentrations were a) 0μM, b) 2μM, c) 20μM, d) 50μM, e) 100 μM. The track labelled 'G' is a Maxam-Gilbert marker lane specific for guanine.

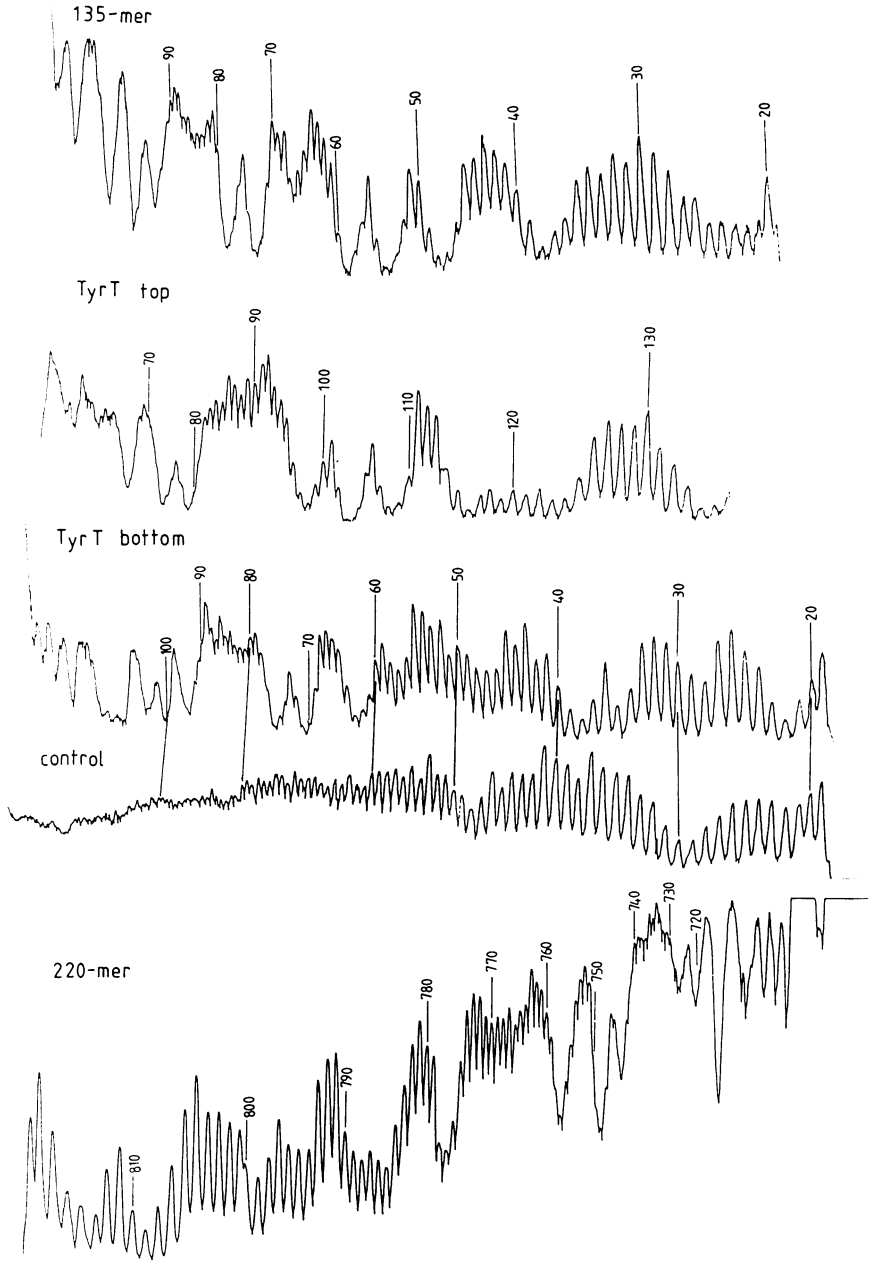

Figure 5. Densitometer scans of hydroxyl radical footprints for 50μM mithramycin. The sequence numbers correspond to those shown in Figure 6. For the bottom strand of tyrT DNA both the drug-treated and control scans are shown. for the other DNA fragments only the drug-treated patterns are presented; cleavage in the control produces a similarly even ladder of bands, see for example ref. [16].

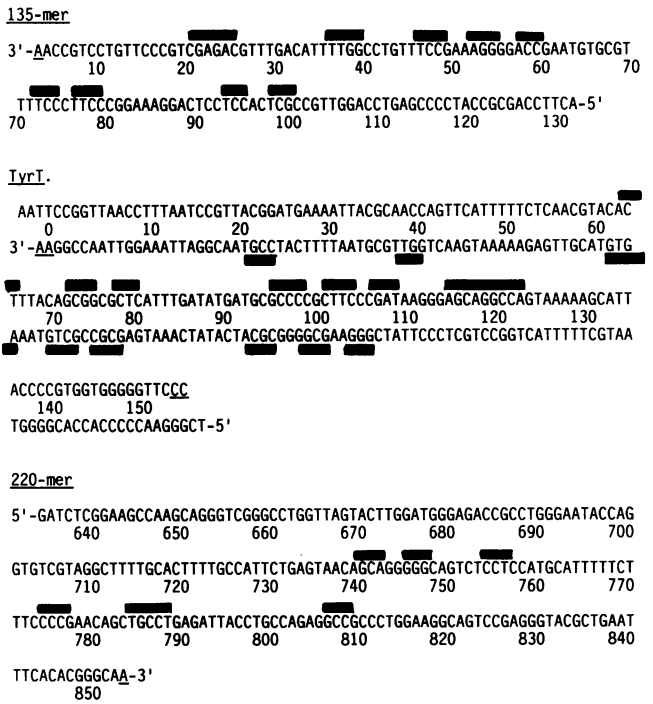

Figure 6. Sequences of the three DNA fragments used. Only the strand bearing the radioactive label (underlined) is shown. The bars represent mithramycin binding sites as determined by hydroxyl radical footprinting.

in which the sequence is located. The suggestion that chromomycin binds as a dimer in an abnormally wide minor groove might indicate that those GC sequences which are located in a DNA regions possessing an unusually narrow minor groove may not represent good binding sites. Before we can rigorously interpret the footprinting data it will therefore be necessary to learn much more about local DNA structure and the ways in which this is influence by surrounding sequences. This may be especially relevant when we consider any structural changes that may be forced onto adjacent regions by ligand binding (see below).

The observation that for several long runs of G and C two mithramycin binding sites can be detected with a more weakly protected region in between was unexpected, especially since such regions tend to possess a wide minor groove and should present good binding sites throughout their length. Possibly the best explanation of this effect is that several potential overlapping drug binding sites are present but that there is sufficient space to accommodate either one mithramycin in the centre or two drugs located at either end. On the basis of probability, if all the sites have equal affinity for the ligand then the most likely configuration is the one with two drug molecules bound. As a result there will be a central region which is only protected by the ends of adjacent molecules and will therefore not appear as a good binding site. This lends weight to the argument that mithramycin does not discriminate between GC-rich regions on the basis of their sequence orientation, but rather according to their structural characteristics.

DNA structural changes. It has been previously demonstrated that mithramycin binding can render certain sequences (especially runs of A and T) more sensitive to cleavage by DNAase I [12]. This has been explained by invoking the theory that the drug opens up the DNA minor groove so that DNA sequences which are normally resistant to nuclease attack, by virtue of their narrow groove width, are rendered more susceptible. Can such an effect be detected by hydroxyl radical cleavage as well? Although hydroxyl radicals produce a fairly even ladder of bands, careful analysis of cleavage patterns has demonstrated that this probe is also sensitive to the DNA local structure [25]. Inspection of the control pattern for the bottom strand of tyrT DNA presented in Figure 5 reveals fluctuations in the otherwise even ladder of bands with minima around positions 30 and 50, both of which correspond to runs of A and T residues, which are known to possess a narrow minor groove [26]. If mithramycin does open up the minor groove as predicted, making it more A-DNA like, then surrounding sequences should be more reactive towards hydroxyl radicals. This is difficult to assess precisely, since measuring such small enhancements is critically dependent on accurate comparisons with suitable control lanes. However it is tempting to suggest that certain regions do indeed display slightly enhanced cleavage in the presence of the antibiotics, for example at positions 87–89, 61–63 and 41–43 on the 135mer, and around position 30 on TyrT DNA.

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