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Sequence-specific binding of luzopeptin to DNA

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ABSTRACT
    We have examined the binding of luzopeptin, an antitumour
antibiotic, to five DNA fragments of varying base composition.
The drug forms a tight, possibly covalent, complex with the DNA
causing a reduction in mobility on nondenaturing polyacrylamide
gels and some smearing of the bands consistent with
intramolecular cross-linking of DNA duplexes. DNAaseI and
micrococcal nuclease footprinting experiments suggest that the
drug binds best to regions containing alternating A and T
residues, although no consensus di- or trinucleotide sequence
emerges. Binding to other sites is not excluded and at moderate
ligand concentrations the DNA is almost totally protected from
enzyme attack. Ligand-induced enhancement of DNAaseI cleavage
is observed at both AT and GC-rich regions. The sequence
selectivity and characteristics of luzopeptin binding are quite
different from those of echinomycin, a bifunctional intercalator
of related structure.

INTRODUCTION
    The luzopeptins (formerly known as BBM 928) are a novel class

of antitumour antibiotics containing two substituted quinoline

chromophores linked by a cyclic decadepsipeptide [1,2].

Luzopeptin A (Figure 1) diplays significant antitumour acivity in

a variety of experimental animal tumour systems [3]. These

antibiotics are closely related to the quinoxaline family, which

includes echinomycin and triostin [4], although there are several

significant structural differences: the luzopeptins have ten

amino acids in the cyclic peptide as opposed to eight in the

quinoxaline family, two of which are an unusual cyclic imino acid

possessing a tetrahydropyridazine moiety, their quinoline

chromophores are substituted with both methoxy and hydroxyl

groups, and they lack a sulphur-containing cross-bridge.

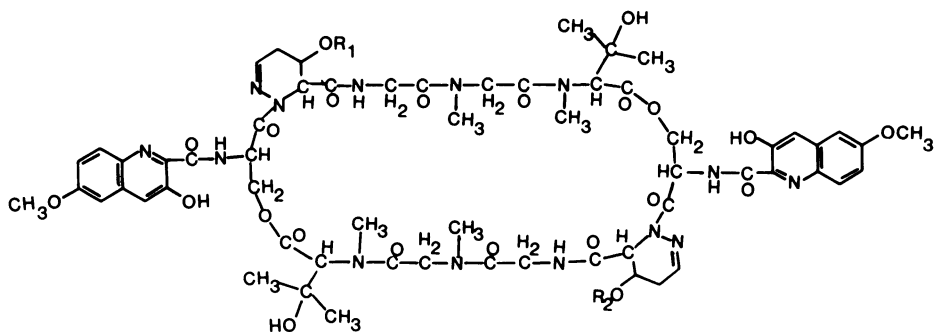   Luzopeptin A has been shown to intercalate bifunctionally

Figure 1. Structure of luzopeptins.
Luzopeptin A $R_1=R_2=COCH_3$
Luzopeptin B $R_1=H$ $R_2=COCH_3$
Luzopeptin C $R_1=R_2=H$

into DNA with one binding site for every five or six base-pairs [1,5]. However, unlike most other bisintercalators it has been reported to form cross-links between DNA duplexes [6]. In another contrast to the quinoxaline family luzopeptin binds strongly to both denatured DNA and RNA [1].

X-ray crystallographic studies have provided details of the three dimensional shape of luzopeptin [7]. The overall structure is similar to that of triostin and echinomycin [8], characterised by a right-handed twist with twofold symmetry. As a consequence of the stereochemistry about the D-serine residues the quinoline chromophores can be aligned in parallel planes orthogonal to the plane of the peptide ring in an appropriate configuration for bifunctional intercalation. Based on this model the interchromophore distance can range from 1.20-1.45nm, which suggests that the molecule could easily span three base pairs, consistent with the binding data if there is neighbouring site exclusion between adjacent bound ligands.

It has been shown that echinomycin binds to GC-rich regions in DNA [9], and recent footprinting studies have confirmed that it binds preferentially to sequences containing the dinucleotide step CpG [10,11]. By contrast, the synthetic quinoxaline depsipeptide TANDEM binds to the step TpA [12]. In this paper we use DNAaseI and micrococcal nuclease footprinting experiments to determine the sequence selectivity of the luzopeptin group of

antibiotics and compare the results with those obtained for other bifunctional intercalators.

MATERIALS AND METHODS

Drugs and enzymes

Luzopeptins A, B and C were gifts from Dr M. Konishi Bristol-Banyu Research Institute, Tokyo, Japan. Echinomycin was provided by Drs H. Bickel and K. Scheibli, CIBA-Geigy Ltd. Basel, Switzerland. Stock solutions of each antibiotic were prepared by direct weighing and, because of their low aqueous solubility, were dissolved in dimethyl sulphoxide (DMSO). These solutions were stored in the dark at 4°C and diluted to working concentration immediately before use. The final DMSO concentration did not exceed 2%, at which level it had no effect on the enzyme cleavage patterns. Under these conditions some of the antibiotic precipitated from solution so that the concentrations referred to may not be exact and should be regarded in the sense of upper limits. Deoxyribonuclease I (DNAaseI) was obtained from Sigma and prepared as a 7200 units/ml stock solution in 0.15M NaCl containing 1mM $MgCl_2$. It was stored at -20°C and diluted to working concentrations immediately before use. Micrococcal nuclease (MNase) was obtained from Pharmacia and prepared as a 2500 units/ml stock solution in 50mM Tris-HCl pH 7.6 containing 2mM $CaCl_2$.

DNA fragments

The 160 base pair tyrT and 166 base pair pTyr2 DNA fragments were prepared and labelled at their 3' ends as previously described [13]. The 135 and 220 base pair DNA fragments were isolated from plasmid pXbs1 [14] by digestion with restriction enzyme HindIII, labelling with $\alpha[^{32}P]$ dATP using reverse transcriptase, and subsequent digestion with Sau3A. The radiolabelled fragments were separated on a 6% polyacrylamide gel. The 320 base pair fragment was similarly obtained from plasmid pBR322; it contains the sequence from the HindIII site (position 29) to the Sau3A site at position 349, and was selectively labelled with $\alpha[^{32}P]$ at the 3' end of the HindIII site.

Footprinting

DNAaseI footprinting was performed as previously described [13]. In several instances the enzyme concentration required to produce a cleavage pattern was larger in the antibiotic-treated samples than in the appropriate controls; this has been noted in the text and figure legends. The protocol for micrococcal nuclease footprinting was as previously published [15].

Gel electrophoresis

The products of enzyme digestion were fractionated on polyacrylamide gels prepared in Tris-borate-EDTA buffer containing 7M urea. Gels (8% w/v) were either 40cm long and 0.3mm thick, or 55cm long and 0.2mm thick incorporating a thickness gradient in the lower portion. Gels were fixed in 10% acetic acid, transferred to Whatman 3MM paper, dried under vacuum and subjected to autoradiography at $-70^\circ C$ with an intensifying screen.

Densitometry

Autoradiographs of the products of DNAaseI digestion were scanned with a Joyce-Loebl Chromoscan 3 microdensitometer to produce profiles from which the relative intensity of each band was measured. The chemical identity of digestion products was assigned by reference to dimethyl sulphate-piperidine tracks specific for guanine. Since micrococcal nuclease cuts the O5' bond whereas piperidine cleaves the O3' bond, bands in the MNase treated lanes (representing labelled fragments lacking a 5' phosphate group) run between 0.3 and 1.5 bonds slower, the difference being most noticeable for the shorter fragments. Intensity data were normalised in terms of fractional cleavage $(f)=A_i/A_t$ as previously described [11-13] where A_i is the area under band i and A_t is the sum of the intensities under all bands in any given gel lane. Effects of antibiotics are expressed in the form of $\ln(f_{antibiotic}-f_{control})$, representing the differential cleavage at each bond relative to that in the control. Positive values indicate bonds at which cleavage is relatively enhanced; negative values indicate drug-induced protection from cleavage.
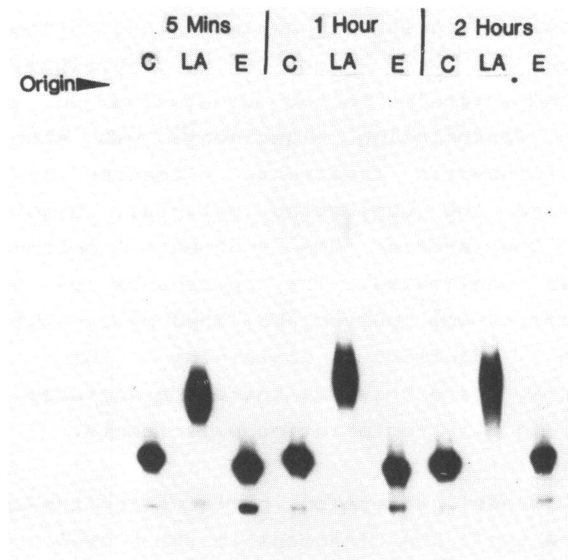
Figure 2. Non-denaturing polyacrylamide gel electrophoresis of tyrT DNA in the absence of antibiotic (C) or after exposure to 20μM luzopeptin A (LA) or 20μM echinomycin (E). The time of incubation is given at the top of each set of three gel lanes.

RESULTS

Before studying the fooprinting patterns produced by luzopeptin the effect of the antibiotic on the mobility of DNA itself was investigated. Samples of luzopeptin-treated DNA were electrophoresed on non-denaturing polyacrylamide gels to produce the results seen presented in Figure 2. Exposure to the ligand reduces the mobility of DNA and causes significant smearing of the bands. These changes appear within 5mins of mixing the drug and DNA, this altered mobility is not changed by longer incubation times. It seems unlikely that these effects could be caused by bisintercalation alone since echinomycin failed to alter the mobility of DNA. Attempts to reverse the effect by phenol extraction and ethanol precipitation were unsuccessful, suggesting that luzopeptin probably remains bound to the DNA by a very strong, perhaps covalent, linkage. This altered DNA mobility

is consistent with some sort of intramolecular DNA cross-linking which has previously been suggested [6], although it could simply be due to very tight DNA binding alone. Likewise, the mobility of DNA molecules in the denaturing polyacrylamide gels employed in footprinting experiments was also occasionally altered by luzopeptin treatment, evidenced by a diffuse band running behind the undigested material. Significantly, the mobility of the shorter DNA fragments produced by enzyme digestion was unaffected. The appearance of molecules with anomalous mobility was however abolished by carefully boiling the samples in solutions containing 1mM NaOH before electrophoresis, and this was therefore included in the enzyme stop solution in all footprinting experiments.

DNAase I footprinting

Typical DNAaseI digestion patterns for the upper and lower strands of the tyrT DNA fragment in the presence of luzopeptins or echinomycin are shown in Figure 3. It is immediately apparent that the luzopeptins alter the digestion pattern in a concentration-dependent fashion and that the results obtained are very different from those seen with echinomycin. In the presence of 20μM luzopeptin there is widespread protection against cleavage, with few bands remaining visible, suggesting that the ligand is able to bind to many sites along the DNA. With 2 and 5uM antibiotic the pattern is intermediate between the control and 20μM. At 2μM ligand no blockage sites can be identified, although the cleavage pattern is altered relative to that in the control, witness the enhancements at positions 28 and 47 (Figure 3a). The digestion patterns observed in the presence of luzopeptins B and C are similar those seen with luzopeptin A except that B and C appear to afford more efficient protection. The pattern produced by 5μM luzopeptin C is characterised by the widespread protection comparable to the effect of 20μM luzopeptin A, so that in the autoradiograph presented in Figure 3b little enzyme cutting can be seen. Each gel lane contains about 100 reasonably well resolved bands which were analysed as described in the Methods section. The results for both strands are presented in the form of a differential cleavage plot in Figure 4. Six major protected regions can be discerned, located
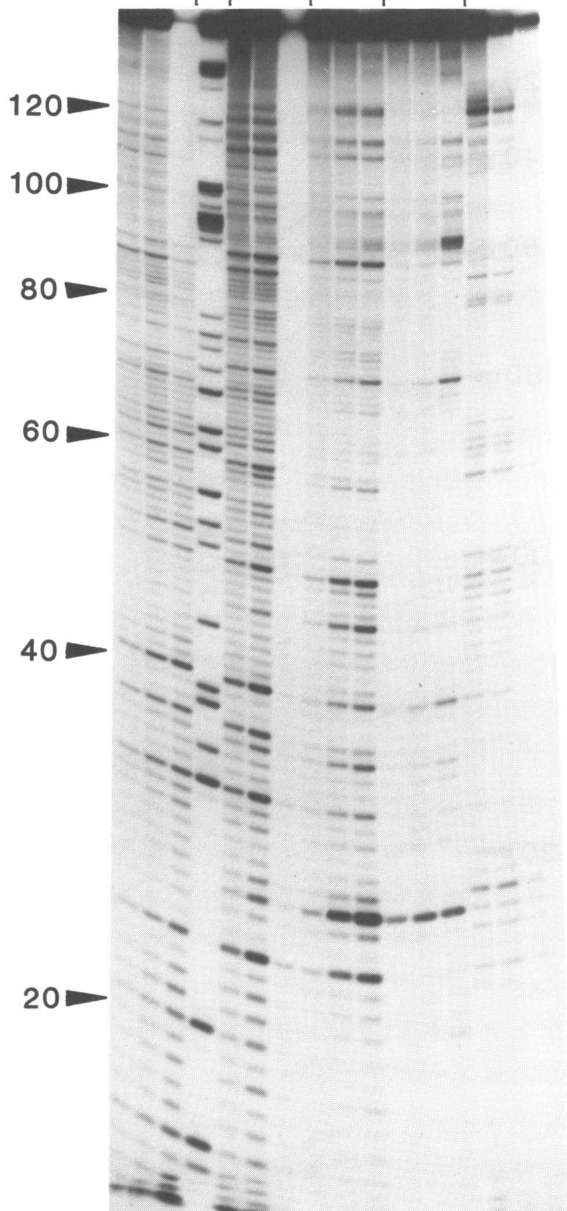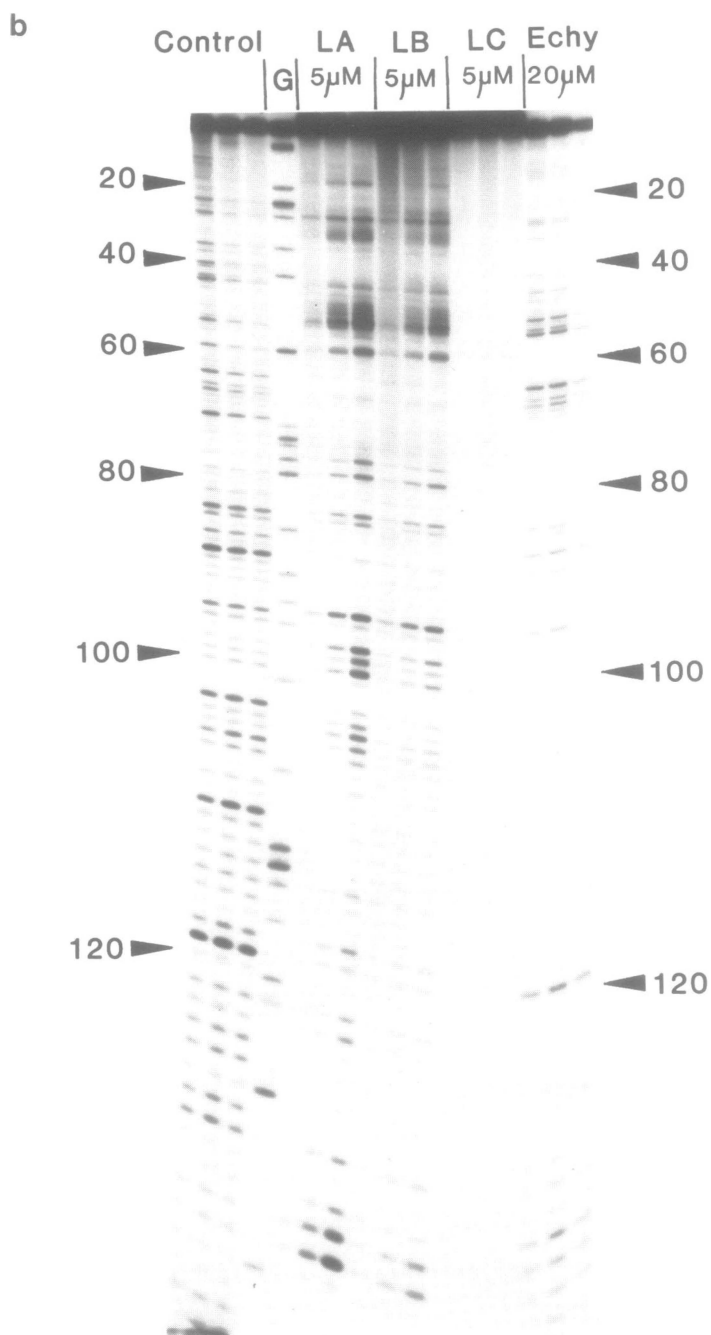
around positions 20, 65, 88, 117, 126, and 135. The size of these protected sites varies from 4 to 12 bases, although the latter (around position 65) could well represent two overlapping binding sites. On searching for a base sequence common to all the protected regions it is noticeable that all are close to the dinucleotide step TpA, although not all such steps are protected. Luzopeptin-enhanced sites of cleavage are also apparent in certain regions, most notably around positions 28 and 47, both of which contain oligomeric runs of A and T. Enhancement of cleavage at these sites has previously been observed with both echinomycin and actinomycin D [11,16].

In order to clarify and extend these observations similar DNAaseI footprinting experiments were performed with luzopeptin A using four other DNA fragments. Footprinting patterns for both labelled strands of pTyr2 DNA are shown in Figure 5 along with the corresponding differential cleavage plot in Figure 6. Further differential cleavage plots determined with the 135mer, 220mer and 320mer DNA fragments are presented in Figure 7. With pTyr2 DNA the pattern produced by luzopeptin is again very different from that seen with echinomycin: three major regions are protected from cleavage centred around positions 72,85 and 122. A possible fourth site can be discerned near position 36 on the lower strand. All of these binding sites are close to the dinucleotide step TpA. A pronounced enhancement is apparent in the run of A and T nucleotides around position 62. The 220mer contains at least five well-resolved binding sites

Figure 3. DNAase I footprinting of luzopeptin and echinomycin on the 160 base pair tyrT DNA fragment. Each set of three lanes corresponds to digestion by the enzyme for 1, 5, and 30 minutes. Tracks labelled "G" are dimethyl sulphate-piperidine markers specific for guanine. (a) DNA labelled at the 3' end of the lower (Crick) strand. The concentration of each antibiotic is shown at the top of each gel lane. The enzyme concentration was increased threefold for 2 and 5μM luzopeptin and sixfold for 20μM luzopeptin. (b) DNA labelled at the 3' end of the upper (Watson) strand in the presence of 5μM luzopeptin A (LA), 5μM luzopeptin B (LB), 5μM luzopeptin C (LC) and 20μM echinomycin (Echy). The enzyme concentration employed was threefold higher in the luzopeptin treated samples than for the control and echinomycin lanes.
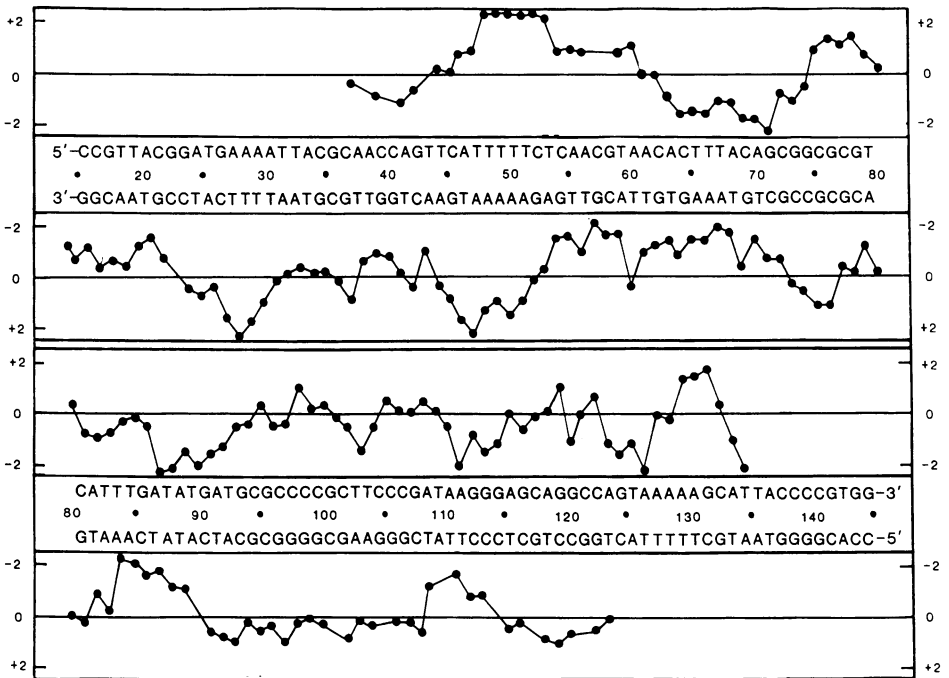
Figure 4. Differential cleavage plot showing differences in the relative probability of cutting of tyrT DNA by DNAase I in the presence of 5μM luzopeptin A . Negative values indicate drug-induced protection, positive values indicate enhancement (log scale).

around positions 732, 760, 780, 795 and 818 , only one of which (at position 795) is associated with the step TpA. Interestingly, the major sites of enhancement in this DNA fragment, which is unusually GC rich, occur around positions 745 and 777 in oligomeric runs of G or C residues. With the 320mer few regions of decisively altered cleavage are apparent although protection can be discerned at positions 96, 122, 135, and 145. A strong enhancement is also present in the run of C residues around position 172. The 135mer presents 3 major sites of protection in the vicinity of positions 30, 60 and 90 with a strong enhancement in the run of thymine nucleotides around position 35.

Micrococcal nuclease footprinting

Typical MNase digestion patterns for the tyrT DNA fragment

in the presence and absence of luzopeptin A are presented in
Figure 8. Protected regions can be identified on both strands
around positions 60-66, 87, 109-111. Longer blockages are visible
on the upper (Watson) strand which include positions 32-33 and
123-125. Most of these regions are similar to those protected
from DNAaseI attack and confirm the identity of the ligand
binding sites on this DNA fragment. Other regions of enhanced
cleavage can also be seen, especially around positions 30 and 50.
These are ach in homopolymeric runs of A and T residues, which
have previously been shown to be relatively refractory to MNase
cleavage [15]. It appears then that luzopeptin can render these
regions more susceptible to attack, in a similar fashion to the
enhancements seen with DNAaseI in the same regions.

DISCUSSION
     The results presented in this paper demonstrate that
luzopeptin binds to DNA in a sequence-selective fashion and that
its recognition properties are very different from those of the
related antibiotic echinomycin. Before considering the nature
of the preferred binding sites for luzopeptin we will first
discuss the mode of binding of this antibiotic, since this will
have implications for the interpretation of the footprinting
patterns.
Mode of binding
     The observations which led to the experiment of Figure 2.
confirm the previous suggestions that luzopeptin does not behave
as a conventional bisintercalator [6]. The reduced mobility of
antibiotic-treated DNA fragments and the smearing of DNA bands
are effects peculiar to luzopeptin and must somehow reflect the
way in which this ligand is bound to the DNA. The altered
mobility could arise either from some form of cross-linking
between DNA molecules or merely from very tight bisintercalation
causing an increase in the length and rigidity of the DNA.
Echinomycin does not cause any such retardation, but this could
be simply because the antibiotic dissociates from the DNA during
the running of the gel. The observation that the retarded DNA
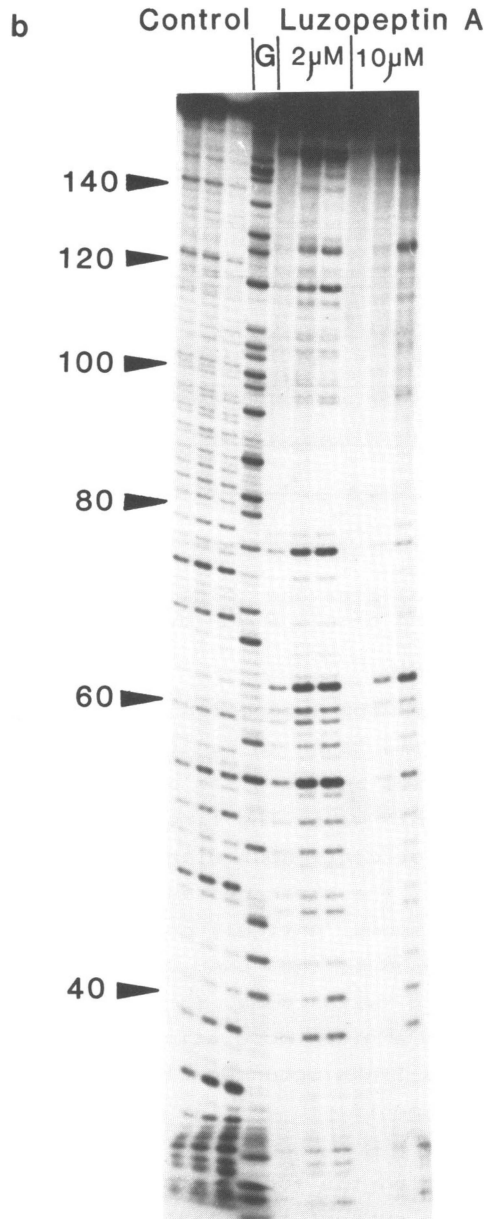band is smeared on the gel could again be explained in two

b   Control Luzopeptin A
         |G| 2μM |10μM

140 ▶

120 ▶

100 ▶

80 ▶

60 ▶

40 ▶
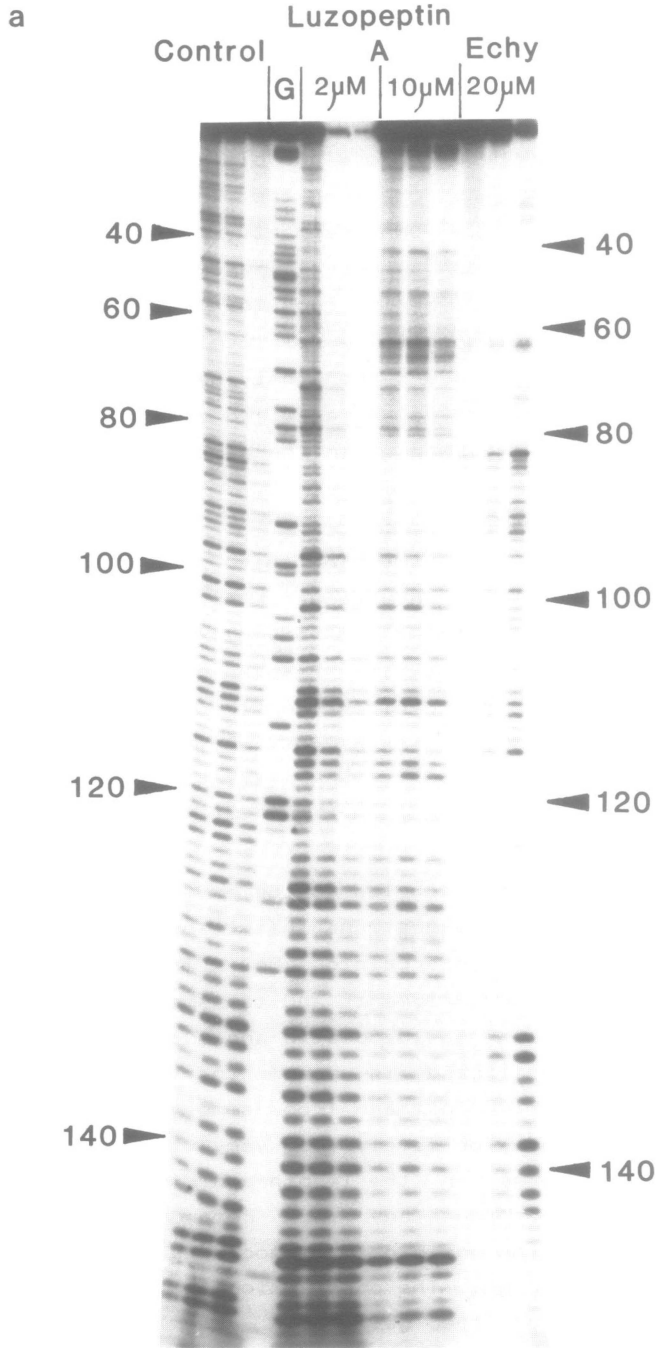
Figure 5. DNAaseI footprinting of luzopeptin and echinomycin on the pTyr2 DNA fragment. Gel tracks are labelled as described in the legend to Figure 3. (a) DNA labelled at the 3' end of the upper strand. The enzyme concentration was 10-fold higher in the luzopeptin-treated samples than the control and echinomycin lanes. (b) DNA labelled at the 3' end of the lower strand. Same conditions as for (a).
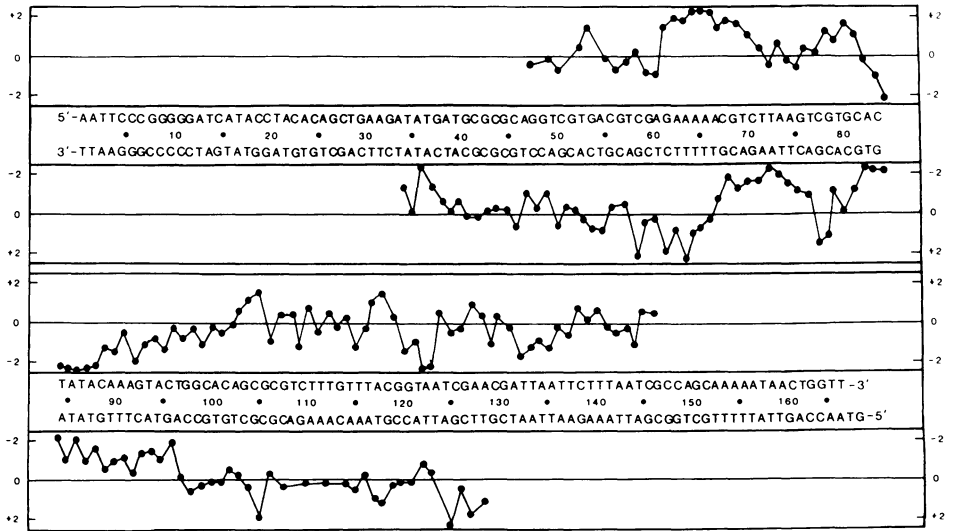
Figure 6. Differential cleavage plot representing the relative probability of cutting of pTyr2 DNA by DNAase I in the presence of 5μM luzopeptin A.

different ways: either the luzopeptin is slowly dissociating from the DNA during the time taken to run the gel or the modified DNA is not a unique species and consists of drug and duplex complexed in several different ways. The first suggestion seems unlikely since the smear is not continuous with the untreated control DNA. It seems likely that the smear represents a Poisson distribution of bound ligand. It seems entirely possible that a proportion of the antibiotic is bound to the DNA via a covalent linkage. The data are consistent with some form of cross-linking reaction which would generate a mixed population of DNA species depending on whether the cross-links occurred close to the centre or the ends of the DNA fragments.

Sequence selectivity

At first inspection the footprints left by luzopeptin on both tyrT and pTyr2 DNAs suggest that the ligand may recognize the dinucleotide step TpA. However, not all such steps are protected and, when considered together with the results obtained with the other three DNA fragments, such a simple interpretation seems unlikely. A dinucleotide step may also be unlikely as a
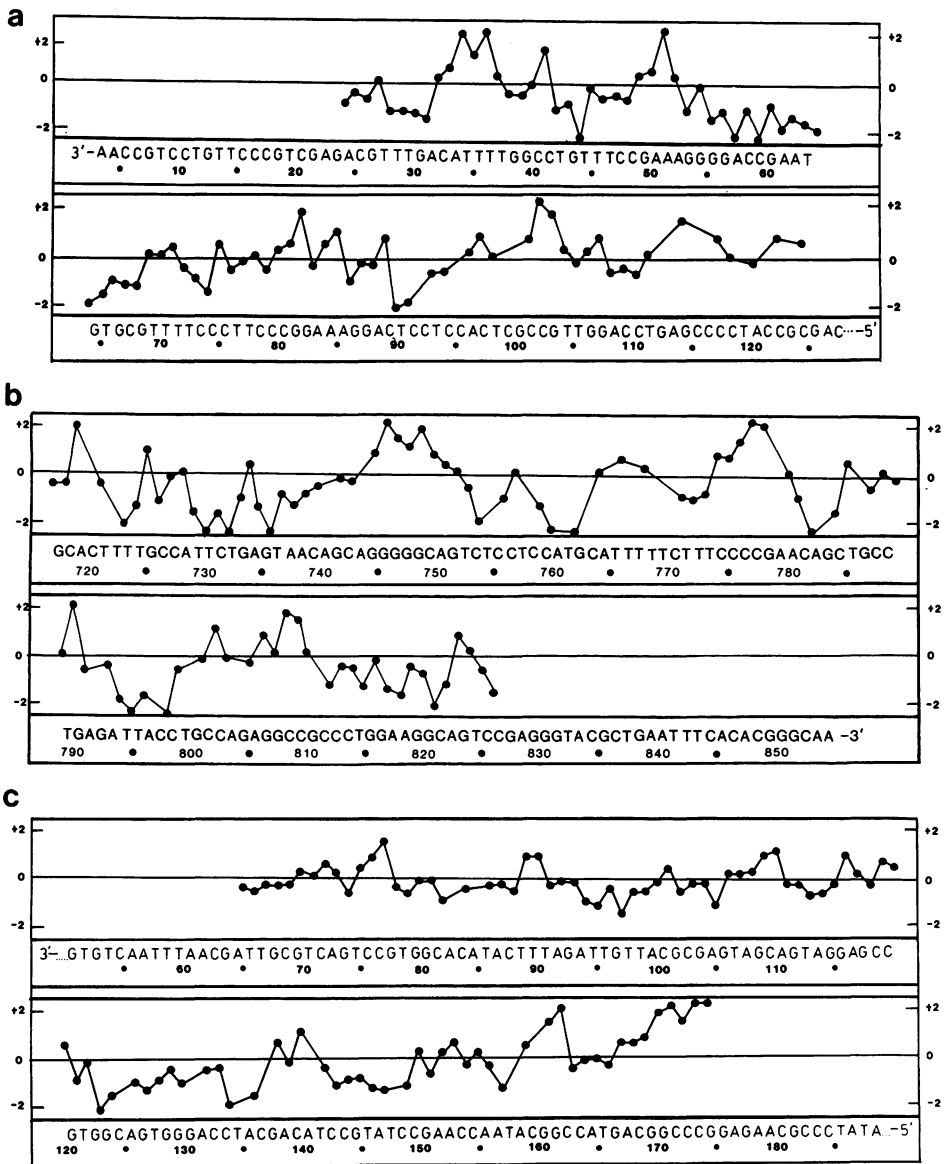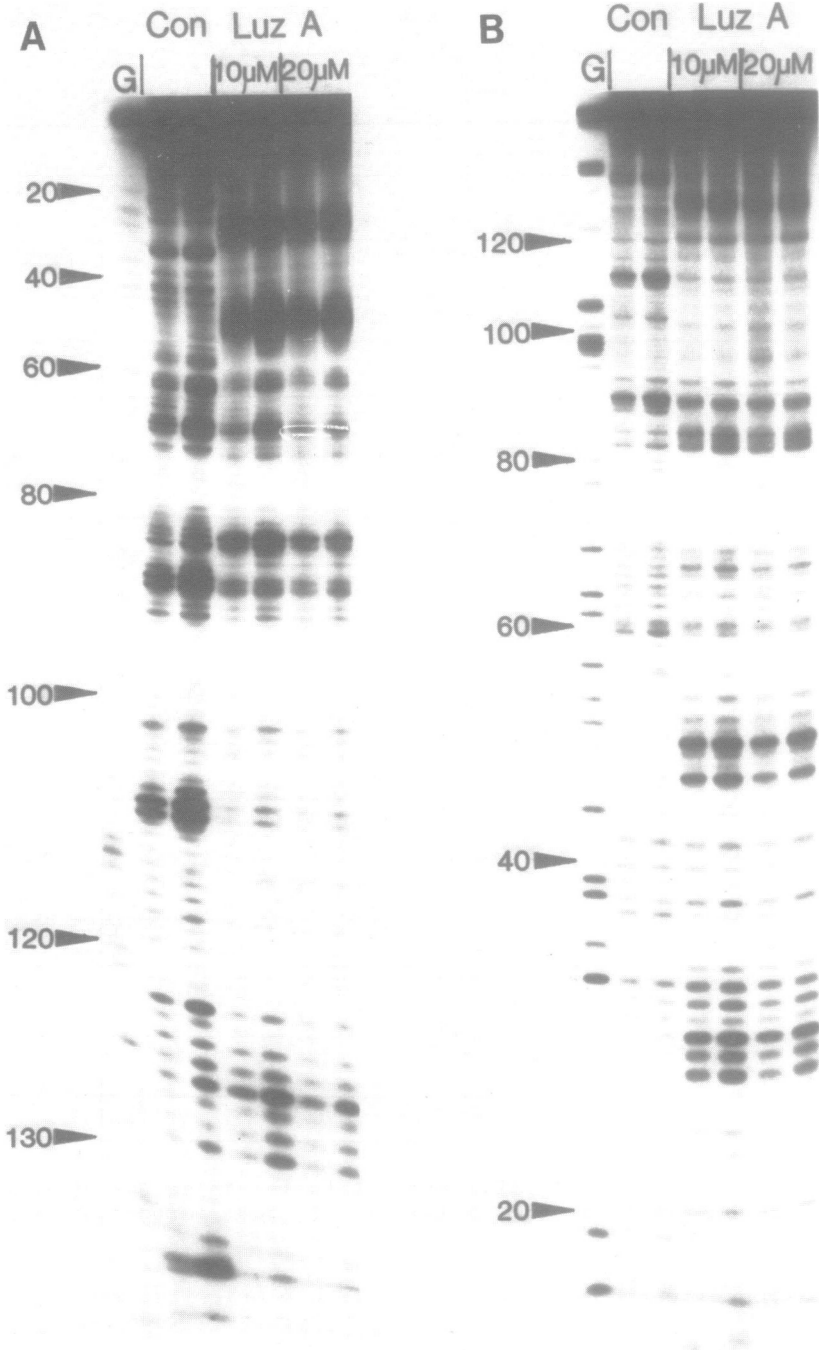
Figure 7. Differential cleavage plots for three DNA fragments exposed to DNAase I in the presence of 5μM luzopeptin A. (a) 135mer. (b) 220mer. (c) 320mer.

recognition site for a symmetrical molecule which can span three base pairs. The symmetry in the structure of the ligand implies that the preferred binding site should also be symmetrical,

and if it consists of a specific arrangement of three base pairs then there are eight possible trinucleotide steps (AAT, AGT, TGA, TTA, CGG, CTG, GTC, GCC) which could fit the bill. No single one of these sequences describes all the binding sites observed in any one DNA fragment let alone all five DNA species so that such a simple interpretation is ruled out. The actual state of affairs is likely to be even more complicated if the suggestion is true that luzopeptin may possess more than one binding mode. Intermolecular cross-linking of DNA strands would also be expected to produce long protected regions since enzyme cleavage will be blocked not only by the offending ligand but also by the second opposing double-stranded DNA molecule. In this context it is worth noting that at the highest drug concentrations employed (20µM) almost no cleavage of the DNA was detected. This behaviour distinguishes luzopeptin from echinomycin which induces a specific pattern of protection in an all-or-none fashion [11] in which no protection is visible below 5uM echinomycin, and the pattern of cleavage remains constant above 10uM ligand. The dramatic change in luzopeptin footprinting pattern over such a narrow range of antibiotic concentration argues in favour of a cross-linking reaction rather than sequence-specific binding to different sites which are occupied according to their relative affinities. Alternatively it is possible that this arises from a longe range drug-induced change in DNA conformation.

What then are the sequence recognition characteristics of this ligand? It appears that luzopeptin does not possess any absolute sequence binding requirements although regions protected from enzyme attack are generally rich in A and T residues. Runs of contiguous A or T nucleotides do not constitute good binding sites and often appear as regions of enhanced cleavage. The results with micrococcal nuclease on the tyrT fragment

Figure 8. Micrococcal nuclease digestion patterns of tyrT DNA in the presence and absence (CON) of luzopeptin A. The concentration of antibiotic used is shown at the top of the appropriate gel lanes. Each pair of lanes corresponds to digestion by the enzyme for 1 and 5 minutes. Tracks labelled "G" are dimethyl sulphate-piperidine markers specific for guanine. (a) DNA labelled at the 3′ end of the upper (Watson) strand. (b) DNA labelled at the 3′ end of the lower (Crick) strand.

confirm that the apparent binding sites are real and are not merely an artefact of using DNAaseI. A more precise definition of the antibiotic binding site(s) on DNA must await more information concerning the precise structure of the luzopeptin-DNA complex (or complexes) by X-ray crystallography or NMR.

Structural changes

A common feature of sequence-selective drug binding to DNA is the occurrence of enhanced enzyme cleavage in regions surrounding some of the ligand binding sites. Echinomycin, actinomycin and nogalamycin induce distinct patterns of enhanced DNAase I cleavage which are located in runs of A and T nucleotides [11,13,16] whereas antibiotics of the distamycin group produce enhancements in GC-rich regions [16]. Binding of luzopeptin produces unusual results in that on different DNA fragments strong DNAaseI enhancements are found in both AT-rich and GC-rich regions. This is unexpected since enhancement of cutting at AT-rich regions has previously been interpreted as resulting from a local widening of the DNA minor groove whereas enhancement at GC residues is thought to arise from narrowing the minor groove. How can luzopeptin do both? This apparent contradiction may be taken as further evidence that luzopeptin has more than one binding mode and perhaps can intercalate from either the major or the minor helical grooves. It is worth noting that the three clearest examples of luzopeptin-induced enhancement in GC-rich regions (positions 745 and 765 on the 220mer and position 100 on the 135mer) are adjacent to, and presumably induced by, antibiotic binding sites which are not conspicuously AT-rich (AGTC, ACAG and ACTC respctively). It is tempting to speculate that when the antibiotic binds to an AT-rich region it causes a local helical distortion rendering neighbouring homopolymeric runs of A and T more susceptible to cleavage, whereas binding to other sites causes the opposite structural effect and facilitates cleavage in GC-rich tracts.

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