FEBS Letters 420 (1997) 156–160 FEBS 19657

Small ligands that neither bind to nor alter the structure of $d(GA{\cdot}TC)_n$ sequences in DNA

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Received 14 November 1997

Abstract Three minor-groove binding ligands have been used to study the characteristics of two $d(GA{\cdot}CT)_n$ DNAs embedded in longer DNA fragments. The binding of mithramycin, netropsin or Thia-Net to these sequences has been studied using DNAse I footprinting. None of these ligands appeared to bind to $d(GA{\cdot}CT)_5$ nor to $d(GA{\cdot}CT)_{22}$ extensively, although with mithramycin some protected bonds were detected at the very edge of these sequences. In general, these small ligands did not enhance the DNAse I cleavage patterns at the alternating $d(GA{\cdot}CT)_n$ flanking sequences located near DNA regions where the drug was bound. The $d(GA{\cdot}CT)_n$ sequences could act as a rigid block in which it is not easy to propagate structural changes, whereas other sequences flanking the binding sites showed cleavage enhancements.

Key words: $d(GA{\cdot}TC)_n$ sequence; DNA-drug interaction; Mithramycin; Netropsin; Thia-Net

1. Introduction

Alternating $d(GA{\cdot}CT)_n$ DNA sequences are quite abundant in eukaryotic genomic DNA [1,2]. These sequences show an outstanding degree of polymorphism that depends on environmental conditions [3–6]. They are frequently found at recombination hot-spots [2], they can arrest DNA synthesis [7], and they play a role in the increased genomic instability in SV40 viruses [8]. Moreover, alternating $d(GA{\cdot}CT)_n$ DNA sequences are often located in or near the regulatory regions of many eukaryotic genes [2,9]. Some protein factors appear to recognize $d(GA{\cdot}CT)_n$ sequences [10,11]. Specific molecular recognition by protein factors is crucial in the regulation of cell processes, while d(GA·CT) sequences of different length might be a dubious binding site for some antitumor drugs [12] that bind to the minor groove of DNA. Much effort has been dedicated to the development of drugs and related ligands that could act as a compatible fitting with the base pairs along the DNA [13,14]. Nevertheless, $d(GA{\cdot}CT)_n$ sequences require special consideration since they are remarkably polymorphic [6]. The alternating conformation of this sequence [15], in which DNAse I cuts the ApG and GpA steps differently [12,16], may become a potential handicap for geometrical fitting along the minor groove.

Mithramycin, netropsin and Thia-Net are small compounds that bind to the minor groove of DNA, though with different sequence preferences. Mithramycin binds to GC-rich regions in the presence of equimolar concentrations of Mg^{2+} [12,17,18]. Netropsin binds to AT-rich regions in DNA that contain clusters at least four bases long [19,20] and neither guanine nor cytosine is allowed to interrupt it. Thia-Net is a thiazole analog of netropsin [13,21] that binds better to four base-pair long sites, mainly composed of A and T residues but with a clear acceptance of intrusive G·C base pairs [21].

In this article we examine the interaction of netropsin, mithramycin and Thia-Net with DNA fragments containing $d(GA{\cdot}CT)_5$ and $d(GA{\cdot}CT)_{22}$ sequences. We analyze the ability of these ligands to bind to or modify the alternating structure of this polynucleotide.

2. Materials and methods

2.1. DNA binding drugs and enzymes

Mithramycin was purchased from Sigma, and netropsin from Serva. Thia-Net was a generous gift from Dr. C.B. Bailly (CNRS, Lille, France). The three drugs were stored at −20°C as a 1 mM stock solution in 10 mM Tris-HCl containing 20 mM NaCl. DNAse I was purchased from Boehringer-Mannheim, prepared as 7200 units/ml stock solution and diluted to working conditions immediately before use.

2.2. DNA substrates

$d(GA{\cdot}TC)_5$ and $d(GA{\cdot}TC)_{22}$ were kindly provided by Dr. M.L. Espinás (CSIC, Barcelona, Spain) as an insert in a pUC18 plasmid carrying a modified polylinker [16]. The DNA fragments containing the sequence to be analyzed were cleaved from the plasmid using *Eco*RV and *Xho*I and purified by electrophoresis using 1% agarose gels. The fragments were labeled at the *Xho*I site using the Klenow enzyme and both [α-^{32}P]dATP and [α-^{32}P]dCTP.

2.3. DNAse I footprinting

Samples of the labeled DNA fragment (around 1 μM in base pairs) were incubated with the appropriate drug solution – see Section 3 – at 30°C for 20 min. In the experiments with mithramycin an equimolar concentration of $MgCl_2$ was added to the reaction mixture, since it is required for drug binding [12,17,18]. The samples were then digested (in a final volume of 6 μl) with DNAse I at a final concentration of 0.01 units/ml. The enzymatic digestion was stopped by adding 3 μl of 85% formamide containing 10 mM EDTA and 0.02% bromophenol blue.

2.4. Gel electrophoresis and analysis of the results

The samples were heated at 90°C for 2 min before electrophoresis. The footprints were resolved by high voltage electrophoresis in 90 mM Tris/Borate, 2 mM EDTA (pH 8.3) buffer using 8% polyacrylamide gels containing 8 M urea, together with a formic acid-piperidine marker specific for guanines plus adenines. After running, the gels were soaked in distilled water, and dried under vacuum and the bands were observed by autoradiography. Analysis of the autoradiographs was performed using a Molecular Dynamics computing densitometer.

3. Results and discussion

Patterns of DNAse I digestion for the DNA fragment containing a $d(GA{\cdot}CT)_5$ insert are shown in Fig. 1. The results correspond to the strand that bears the GA tract. In the con-

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PII S0014-5793(97)01509-3

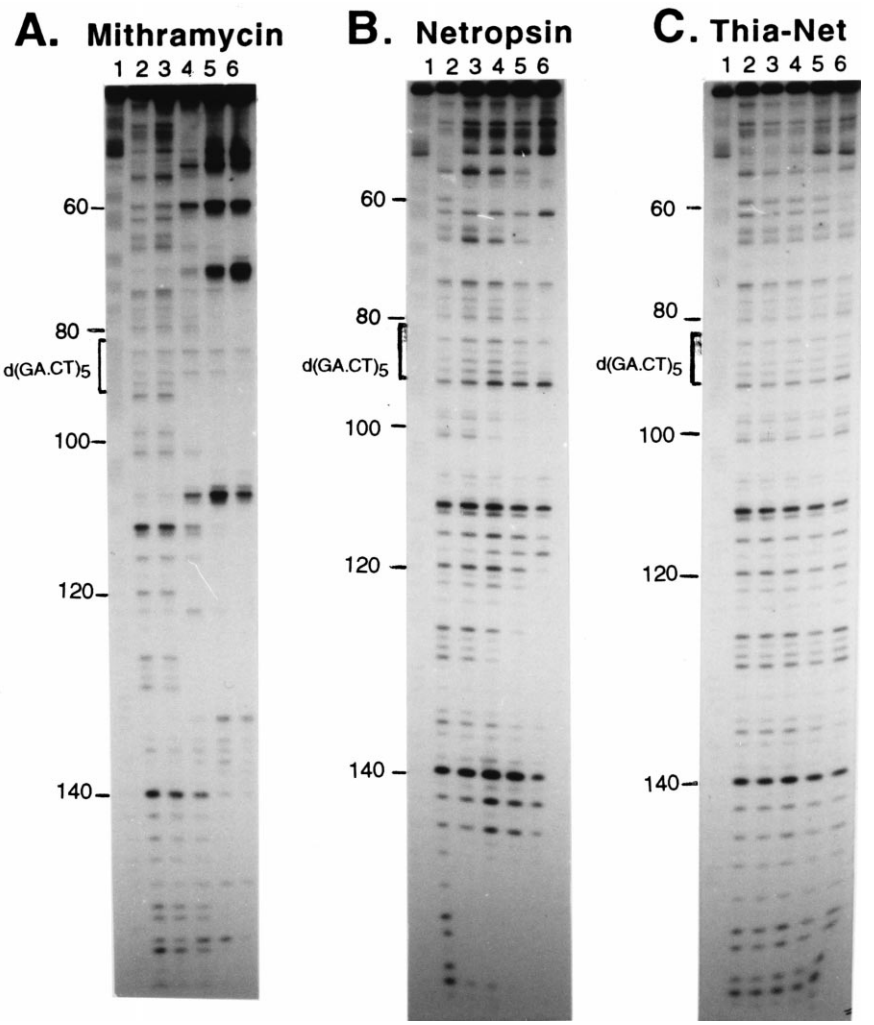

Fig. 1. DNAse I footprinting of mithramycin, netropsin and Thia-Net on a DNA fragment containing a $d(GA{\cdot}CT)_5$ insert whose sequence is shown in Fig. 3A. Panel A: (lane 1) A+G sequence ladder; (lane 2) control, no drug; (lanes 3–6) 1, 5, 10, 50 μM mithramycin. Panel B: (lane 1) A+G sequence ladder; (lane 2) control, no drug; (lanes 3–6) 1, 5, 10, 15 μM netropsin. Panel C: (lane 1) A+G sequence ladder; (lane 2) control, no drug; (lanes 3–6) 1, 5, 10, 50 μM Thia-Net.

trol lanes (no added drug) the DNAse I digestion showed a clear preference for cutting ApG over GpA. It is immediately apparent that the cleavage patterns in the presence of the ligands mithramycin (panel A), netropsin (panel B) or Thia-Net (panel C) were substantially different from those of the DNA alone. Nevertheless, the preference for cutting ApG bonds over GpA in the large d(GA·CT) sequences was always sustained. The footprints in regions other than the $(GA)_5$ inserts were largely corroborative of the binding selectivity of the three ligands to DNA, previously analyzed [12,18–21]. A summary map showing the difference in susceptibility of this DNA fragment to DNAse I in the presence of the higher concentrations of the three ligands is displayed in Fig. 3A. Fig. 1A presents DNAse I footprinting patterns of the DNA fragment in the presence of increasing concentrations of mithramycin. With 1 μM mithramycin the patterns of DNAse I digestion remained essentially as in the control lane. Higher concentrations (5–10 μM) produced clear sites of drug protection that can be discerned along the DNA fragment. Moreover, there are clear enhanced bands around them. At higher concentrations (50 μM) a new protected region appeared between bonds 140 and 152 and the region around 120 became wider. The protected region close to the $d(GA{\cdot}CT)_5$ insert might reflect the strong binding to the GGG tract at the very edge of the GA tract, as substantiated by the enhancements at the 109–111 nucleotides. At the higher drug concentrations, the protected region covers about 3 bases of the GA insert (bases 89–91). It is worth mentioning that because DNAse I is a large molecule the sequence protected from the enzymatic attack cannot be straightforwardly correlated to the exact binding site [19,22]. The protected regions in Figs. 1 and 3A might correspond to an indirect effect due to the accommodation in the minor groove of one of the saccharide chains of mithramycin, rather than the direct recognition of the sequence.

Visual inspection of the patterns of DNAse I cleavage in the presence of different concentrations of netropsin, Fig. 1B, revealed several well-resolved protected regions that contain AT-rich sequences. Neither of these protected regions covered the $d(GA{\cdot}CT)_5$ region, although concentrations of 10–15 μM netropsin produced clear footprints in the neighboring regions. At 15 μM the cleavage of some bonds at the middle

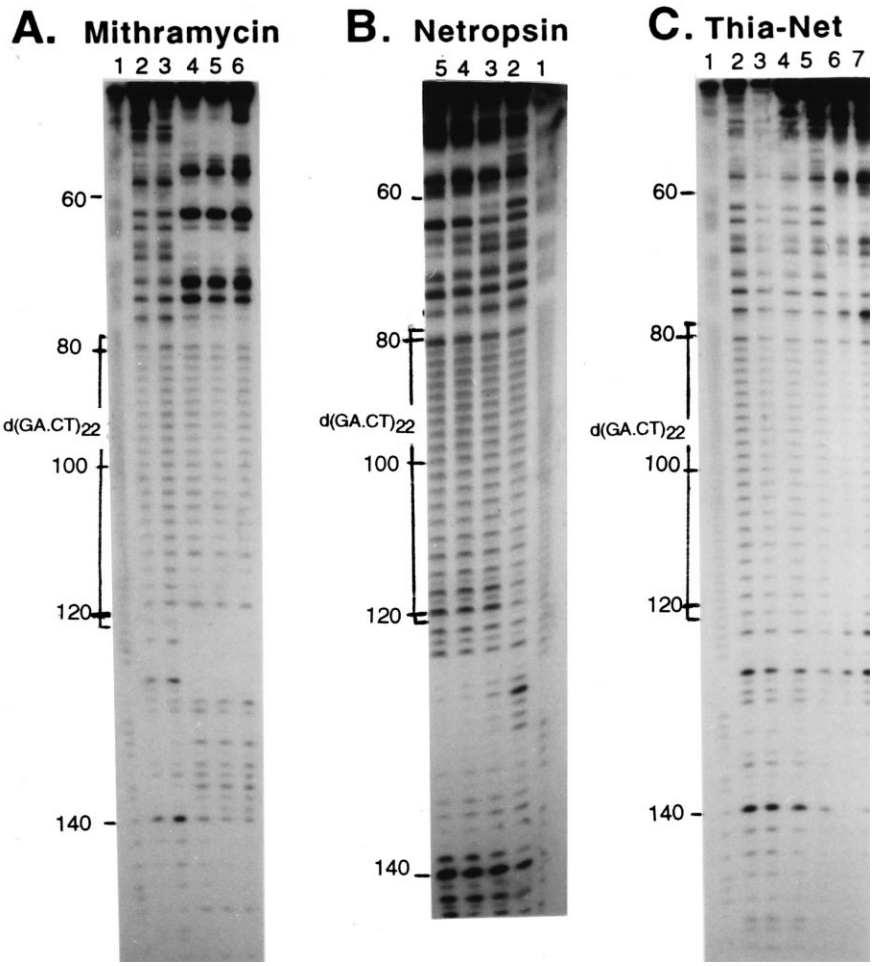

Fig. 2. DNAse I footprinting of mithramycin (panel A), netropsin (panel B) and Thia-Net (panel C) on a DNA fragment containing a $d(GA{\cdot}CT)_{22}$ insert whose sequence is shown in Fig. 3B. Panel A: (lane 1) A+G sequence ladder; (lane 2) control, no drug; (lanes 3–5) 1, 5, 10 μM mithramycin. Panel B: (lane 1) A+G sequence ladder; (lane 2) control, no drug; (lanes 3–6) 1, 5, 10, 15 μM netropsin. Panel C: (lane 1) A+G sequence ladder; (lane 2) control, no drug; (lanes 3–7) 1, 5, 10, 50, 100 μM Thia-Net.

of the GA sequence seems to be slightly diminished while the flanking sequences at both sites remained unaltered (see Fig. 3A). A simple explanation for these protected bonds is a weaker, secondary, netropsin binding site, since we do not expect that netropsin could recognize the 2-amino group of guanine [13,20]. The binding of increasing amounts of netropsin produced weak enhancements at the vicinity of some binding sites, while the $(GA)_5$ sequence did not show any symptom of enhanced cleavage.

DNAse I footprinting with this DNA fragment showed three well-resolved Thia-Net binding sites (Fig. 1C) that were detected at concentrations higher than 5 μM. They contain various isolated GA sites (Figs. 1 and 3A) in agreement with previous studies [21]. In any case, Thia-Net had no effect on the pattern of DNAse I cleavage in the $d(GA{\cdot}CT)_5$ sequence. It is likely, therefore, that Thia-Net cannot recognize the alternating helical twist pattern of this sequence [15], though it accepts internal G·C pairs in the binding site [21].

Fig. 2 displays the cutting patterns produced by DNAse I on the DNA fragment containing a $(GA{\cdot}CT)_{22}$ insert, in the presence of different concentrations of mithramycin, netropsin and Thia-Net. A protection map of the effect of the three ligands on this DNA is displayed in Fig. 3B. We shall pay most attention to the possible binding of those drugs to the $d(GA{\cdot}CT)_{22}$ inserts. We study the alterations (enhancements), or binding in the neighboring regions, rather than the well-known binding preferences of these ligands in more sequence-averaged DNAs. In the control lanes (no added drug), the DNAse I cleavage also exhibited the preference for cutting ApG over GpA described above. The presence of any of the three drugs did not significantly alter this pattern of cleavage. At first glance, it seems that these drugs were not bound to the $d(GA{\cdot}CT)_{22}$ tracts. They did not enhance the cleavage at these tracts, nor did they change the differential cutting at the GpA and ApG steps. Broadly speaking, the binding sites that can be identified for the three ligands (Figs. 2 and 3B) corresponded to those previously described on other DNA fragments [12,19,21]. Thia-Net protected regions that contain short AG tracts from cleavage, for example around 130–136 (Fig. 2C), but it did not bind or alter the longer $(GA)_{22}$ tract. Likewise, our results indicate that this drug does not bind to larger tracts containing strictly alternating G·C/A·T base pairs.

The DNAse I footprinting analyzes suggest that mithramycin (Fig. 2A) did not bind to the large GA tract within the DNA fragment (see also Fig. 3B). Notwithstanding, clear

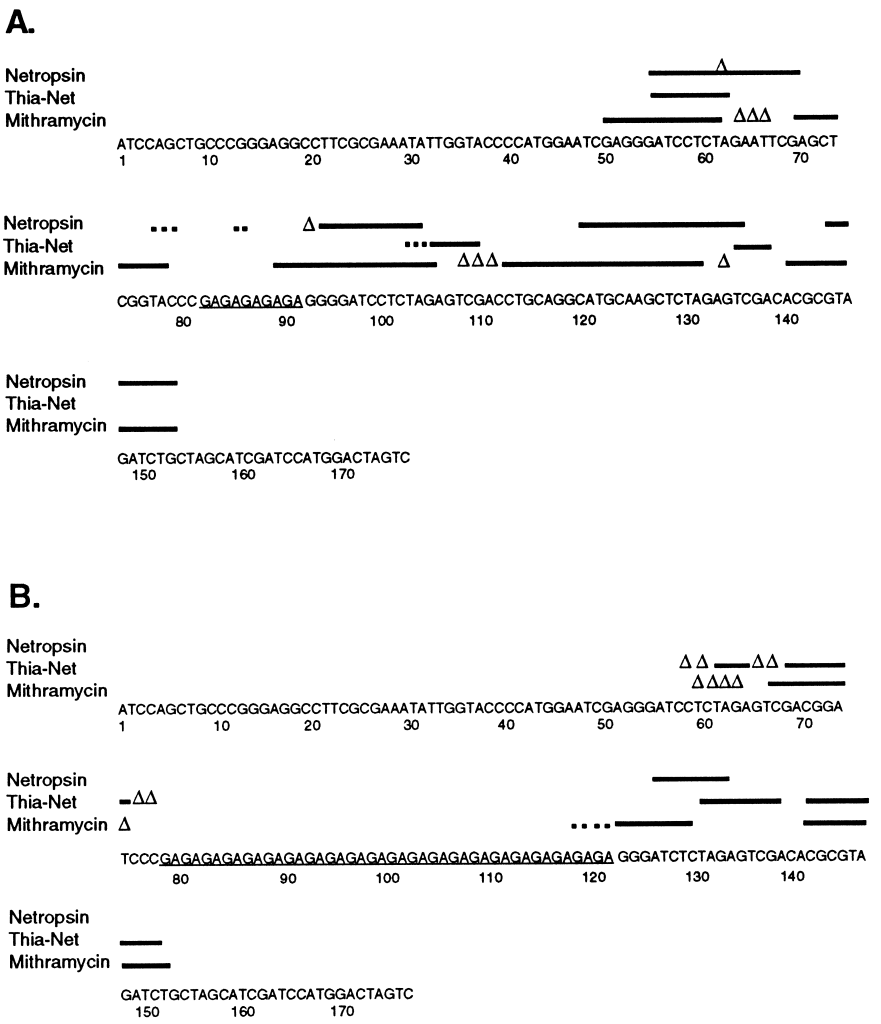

Fig. 3. Protection maps showing the effects of 50 μM mithramycin, 15 μM netropsin, and 50 μM Thia-Net on the DNase I footprinting of a DNA fragment containing a $d(GA{\cdot}CT)_5$ insert (panel A) or of a DNA fragment containing a $d(GA{\cdot}CT)_{22}$ insert (panel B). Bars indicate sequences which are protected from cleavage. Broken bars indicate regions where the extent of protection was either weak or doubtful. Triangles indicate enhanced cleavage in the presence of the ligands.

strong binding sites were detected on both sides of the insert. They correspond to zones containing CG-rich sequences, consistent with the known sequence-selective binding of this drug [12,17,18]. Besides, the cleavage at some flanking regions appeared to be enhanced. The AG insert retained the preference for cutting ApG over GpA as in the control – no added drug – experiments (Fig. 2A). At concentrations over 10 μM, a few bonds close to the ends of the insert were slightly attenuated in agreement with the results described above with the shorter insert. This protected region might correspond to the minor-groove alignment of one of the mithramycin saccharide segments on the flanking sides of the $d(GA{\cdot}CT)_n$ tracts [18]. Unlike the $(GA)_5$ insert, the $(GA)_{22}$ one was mostly refractory to both mithramycin binding and the indirect structural alteration of its structure, in spite of the strong binding in the neighboring zone, which is ratified by the enhancements at other regions around the binding sites (Fig. 2A). NMR studies have shown that mithramycin binding makes the minor groove both wider and deeper [17,18]. The propagation of that change in the minor groove is small and it could somewhat depend on the length of d(GA·CT) tracts.

In general, binding of any of the three ligands produced changes in the susceptibility of the flanking sequences to nuclease attack. Nevertheless, these changes did not take place at the $d(GA{\cdot}CT)_n$ inserts. If we assume that DNAse I cleavage enhancements are mostly, but not exclusively, due to changes in the DNA conformation [23], we should conclude that the strong mithramycin binding at the edge of the d(GA·CT) tracts was not capable of altering its structure extensively. A possible explanation is that the alternating conformation of these tracts [15], or the different structural properties in the individual strands [3,5], could act as rigid block in which it is not easy to propagate any structural change. It should be remembered that these sequences can arrest DNA synthesis [7], and they appear to be nucleosome-free in vivo [9]. However, it is worth noting that $d(GA{\cdot}CT)_n$ sequences of different length form nucleosomes in vitro [16].

Whereas we have identified the broad outlines of the drug binding sites by DNAse I footprinting, it seems more difficult to interpret the small differences seen at the very edge of the d(GA·CT) tracts. We have also employed diethylpyrocarbonate (DEPC) footprinting [24,25] to gain new insights into the structural changes in long d(GA·CT) tracts after drug binding. In the presence of mithramycin, a few bases near the drug

binding sites displayed enhanced reactivity to DEPC, in agreement with a previous report [25]. Notwithstanding, the weak DEPC modification of the long d(GA·CT)$_n$ tracts remained unaltered in the presence of any drug concentration (results not shown).

In summary, none of the three drugs studied here binds extensively to the d(GA·CT)$_n$ sequences, nor alters them significantly. They are minor-groove binding ligands that exemplify different sequence preferences. The analysis of their complexes with DNA containing d(GA·CT) inserts indirectly confirms the peculiar characteristics of this sequence. Although the 2-amino group of guanines plays a key role as regards the location of binding sites for minor-groove binders like those analyzed here [26], the alternating polypurine sequences might, through their peculiar conformation, discriminate between some small ligands. The future design of d(GA·CT)$_n$-reading molecules will demand that the characteristic structure of these sequences be pondered carefully, especially the different conformation around the ApG and GpA steps.

Acknowledgements: We are thankful to Drs. C.B. Bailly and M.L. Espinás for providing us with Thia-Net and the d(GA·CT)$_n$ plasmids respectively. This work was financed by grants from the Spanish DGES (PB 95-0064 and PB 96-812). It has been carried out in the framework of the Centre de Referencia en Biotecnologia of the Generalitat de Catalunya.

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