

Competition between Netropsin and Restriction Nuclease EcoRI for DNA Binding

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Abstract

We find that netropsin and netropsin analogue protect DNA from EcoRI restriction nuclease cleavage by inhibiting the binding of EcoRI to its recognition site. The drug — EcoRI competitive binding constants measured by a electrophoretic gel mobility shift assay are in excellent agreement with the nuclease protection results for the netropsin analogue and in reasonable agreement for netropsin itself. Crystal structures of complexes show that netropsin and EcoRI recognize different regions of the DNA helix and would not be expected to compete for binding to the restriction nuclease site. The large distortions in DNA structure caused by EcoRI binding are most likely responsible for an indirect structural competition with netropsin binding. The structural change in the netropsin binding region induced by EcoRI binding to its region essentially prevents drug association. Given the reciprocal nature of competition, binding of netropsin to a minimally perturbed structure then also makes the association of EcoRI energetically more costly. Since many sequence specific DNA binding proteins significantly bend or distort the DNA helix, drugs that compete indirectly can be as effective as drugs that act through a direct steric inhibition.

Introduction

Many biologically active organic compounds show potent pharmacological effects because of their interference with normal DNA function (reviewed in (1)). Both netropsin and distamycin A, pyrrole-amidine antibiotics, show antibacterial and antiviral activity presumably due to their DNA binding properties. These drugs bind tightly in the minor groove of DNA with a strong preference for dA/dT sequences (reviewed in (2)). It has been suggested that these drugs act by disrupting the interactions between DNA and sequence specific DNA binding proteins that are critical for regulating transcription and replication. Although many, if not most, of these proteins recognize DNA sequences through the major groove, the binding of

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netropsin or distamycin in the minor groove has been observed to preclude the binding of several sequence specific DNA binding proteins, e.g., Cro repressor (3), OTF-1 and NFE-1 transactivating factors (4), and *Antp* and *ftz* homeodomain proteins (5). At the other extreme, however, the binding of a netropsin analogue (1-methylimidazole-2-carboxamide netropsin) does not observably affect the binding of yeast transcriptional activator GCN4 to its recognition sequence (6).

A strong competition between drug and protein for binding to the same DNA sequence can arise in two general ways. The two can directly compete for access to the same groups on DNA, a steric competition. Alternatively, even though the drug and protein may bind to different regions of the same DNA sequence and not physically overlap, an indirect competition may still occur if the bound complexes have distinctly different DNA conformations. Both explanations have been put forward to account for the observed competition. In the case of Cro binding (3), the competition with netropsin was taken as possible evidence for important protein contacts in the minor groove, in addition to the known major groove interactions seen in the x-ray structure. In the other instances (homeodomain peptides (5) and OTF-1 and NFE-1 transactors (4)), the competition was attributed to a conformation change in the DNA structure induced by distamycin binding. Although the x-ray structure of a DNA oligomer-distamycin complex does show some distortion of the DNA helix (7), a DNA oligomer netropsin complex, show a very little distortion of the DNA structure (8,9).

The activities of several restriction endonucleases are inhibited by the presence of netropsin and related compounds (10-13). Currently, EcoRI is probably the best characterized of all restriction enzymes (reviewed in (14)). The crystal structure of a complex between EcoRI and oligonucleotide containing the specific site has been solved (15) and recently revised (16). It has been concluded that the minor groove of the DNA in the complex is clearly exposed to the solvent, whereas the major groove is in intimate contact with the protein (15-17). The EcoRI recognition sequence, GAATTC, is also a strong netropsin binding site. In principle, there is no compelling reason from accessibility for a netropsin bound in the minor groove to prevent the binding of EcoRI that interacts primarily with the major groove. Drug inhibition of enzymatic activity could then be at step subsequent to binding. Nevertheless, we find that both netropsin and a netropsin analogue protect DNA from EcoRI restriction endonuclease digestion by competing with EcoRI for binding to the restriction site. The nuclease digestion protection observed can be described by simple drug binding isotherm equations. The apparent binding constant for netropsin analogue — EcoRI site from nuclease protection is in good agreement with the average binding constant of the analogue to strong DNA sites determined by circular dichroism. The difference in binding constants of netropsin to an EcoRI site and to an NdeI site (GATATC) from the nuclease protection is in good agreement with binding constants to these sequences determined by others using different assays [18]. Direct measurement of drug — EcoRI binding competition is possible using an electrophoretic gel mobility shift assay. The directly measured competitive binding constants are in excellent agreement with the protection assay for the netropsin analogue and in reasonable agreement for netropsin itself.

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Even though netropsin and EcoRI bind to distinct nonoverlapping regions, they can not bind simultaneously to the nuclease specific site. The most reasonable explanation is that the large distortions in DNA structure caused by EcoRI binding are responsible for an indirect structural competition with netropsin binding. Just as netropsin stabilizes the canonical B-form structure in the B- to A- transition of DNA (19) and stabilizes double helical, B family poly(dA) · poly(dT) in preference to the triple stranded poly(dT) · poly(dA) · poly(dT) (20, 21), netropsin binding stabilizes the native, solution DNA structure (as seen in the crystal), a conformation incompatible with EcoRI binding. The design of drugs to compete with specific DNA binding proteins does not have to rely on a direct competition for binding to the same groups on the DNA surface. Many drugs that bind nonintercalatively with DNA interact with the minor groove (2), not the major groove that is recognized by many sequence specific DNA binding proteins. Given that many sequence specific DNA binding proteins significantly bend or distort the DNA helix when bound, drugs that compete indirectly can often be as effective as drugs that compete through a steric inhibition.

Methods and Materials

Materials

Netropsin analogue was a kind gift from Dr. G.V. Gursky (Laboratory of the DNA-Protein Recognition, V.A. Engelhardt Institute of Molecular Biology, Moscow, Russia). The synthesis has been described previously (22), (23). Netropsin was purchased from Boehringer Mannheim. The structures of the netropsin and netropsin analogue are shown in Figure 1. Both drugs were stored dry at 4 °C. Fresh solutions of netropsin and netropsin analogue were prepared from dry samples immediately before each experiment. Drugs were first diluted in small amount of methanol, then aqueous buffer added. The final methanol concentration never exceeded 5%. Each solution was used only during one day. Drug concentrations in these stock solutions were determined spectrophotometrically using a value of 2×10^4 for the molar extinction coefficient at 297 nm.

The plasmid pUC19, PacI linker, and restriction enzymes EcoRI, NdeI, ScaI, BanII, KpnI and SacI were purchased from New England Biolabs and used without further purification. Plasmid DNA concentrations were determined spectrophotometrically using a molar (base pair) extinction coefficient of 1.33×10^4 .

Absorption spectra were obtained with a Shimadzu UV-2101PC spectrophotometer.

Nuclease digestion protection.

Plasmid pUC19, 2686 bp long, linearized with restriction enzyme ScaI (position 2177), was used as substrate in all protection experiments. Complexes of netropsin or netropsin analogue and ScaI linearized pUC19 were prepared by direct titration of DNA solutions with drugs. Complexes were incubated in the dark for 20 min on ice. Restriction enzymes were then added and samples incubated at 25 °C for 30 min. The reaction was stopped by direct ethanol precipitation. We find that this procedure

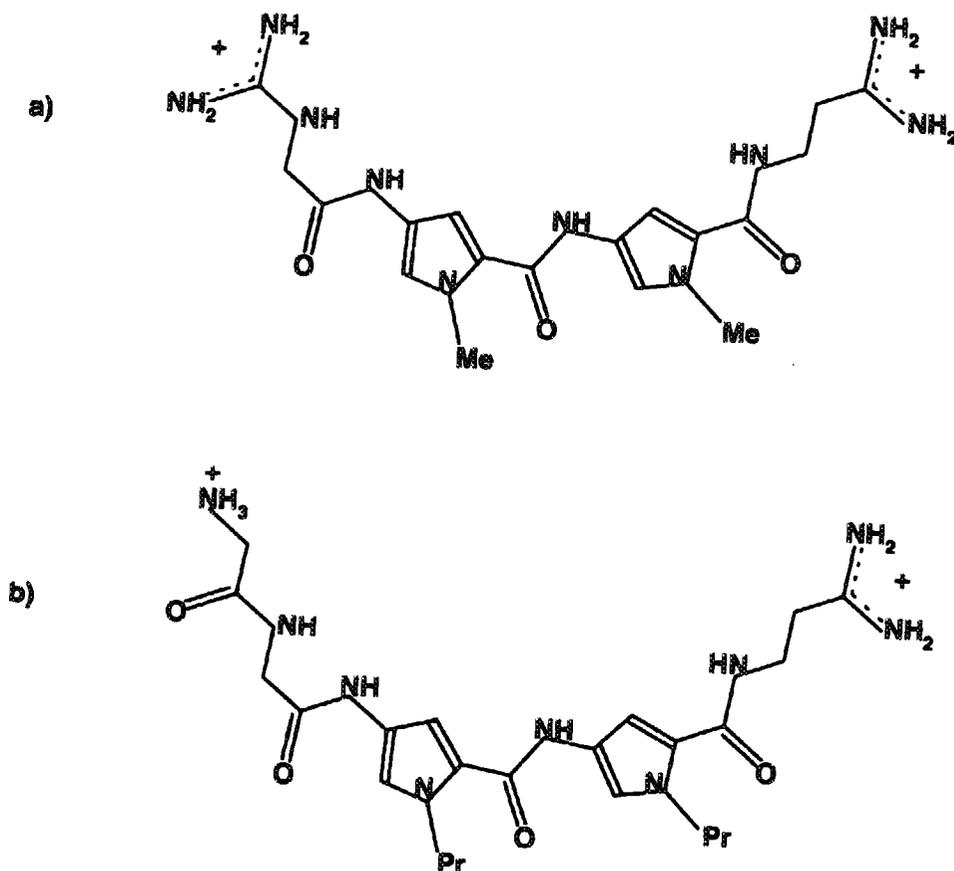
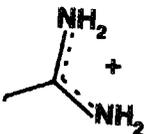
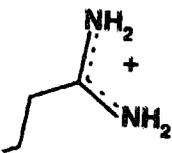


Figure 1: The structural formulas of netropsin (a) and netropsin analogue (b). Me — methyl group; Pr — propyl group.

for stopping the digestion reaction is as effective as the standard method using EDTA. DNA pellets were then collected by centrifugation, washed with 70% EtOH, dried, and resuspended in 25 mM Tris-Cl (pH 7.5), 1 mM EDTA. DNA digestion products were separated on 1% agarose (SeaKem GTG) gels ($0.5 \times$ TBE buffer, at 110 v, 50-54 ma for about 1-1.5 hours).

The salt conditions for the EcoRI protection experiments with netropsin analogue were 45 mM Tris-Cl (pH 7.5), 100 mM NaCl, and 5 mM $MgCl_2$. For protection experiments with netropsin and EcoRI, NdeI, BanII, KpnI, and SacI, a standard salt solution supplied by New England Biolabs (10 mM Tris-Cl (pH 7.9), 0.05 M NaCl, 10 mM $MgCl_2$, and 1 mM DTT) was used. The restriction nuclease concentrations ($\sim 0.5 - 1$ unit/ 100 μ L) were chosen to digest about 60 - 80 % of the plasmid with no added drug under the experimental conditions of salt, temperature, and reaction time used and were determined from pilot titration experiments. We additionally verified that, for fractions of uncut DNA, F, between 10 and 85 %, $\log(F)$ scales linearly with restriction nuclease concentration under these reaction conditions.

The plasmid DNA concentration was typically 1.5 μ M base pairs in a reaction



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volume of 100 μL . Control experiments were performed to confirm that the DNA concentration used was low enough such that the drug bound is small fraction of the total drug added. In these experiments, the DNA concentration was about 0.4 μM bp in a 400 μL reaction volume. tRNA was added to the samples immediately before adding ethanol to enhance the efficiency of DNA precipitation.

Gel mobility shift assay.

A 322 bp fragment, containing the EcoRI recognition site, was isolated from PvuII digested pUC19 using standard agarose gel techniques (24). Complexes of netropsin or netropsin analogue and 322 bp fragment were prepared by direct titration of DNA solutions with drugs. Complexes were incubated in the dark for 20 min on ice. EcoRI nuclease was then added and samples incubated at 25 $^{\circ}\text{C}$ for 30 min in the absence of required Mg^{2+} co-factor. Immediately after incubation samples were loaded on gels. For netropsin analogue competition experiments, the EcoRI binding buffer used was 25 mM Tris-Cl - 5 mM NaCacodylate (pH 7.5), 50 mM NaCl, 25 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, and 2.5 % ficoll. For netropsin competition, the binding buffer was 25 mM Tris-Cl (pH 7.5), 0.6 M NaCl, 25 mM EDTA, 1 mM DTT, 0.1 mg/ml BSA, and 2.5 % ficoll. The DNA concentration was about 5 μM bp (~ 15 nM in EcoRI sites), in 25 μL volumes. Sufficient EcoRI restriction nuclease was added to give about 20-50 % stoichiometrically bound fragment (~ 4 units/ μL).

All gel mobility shift experiments were performed with 1.5% agarose gels, not polyacrylamide as is standard for this type of experiment (25). As has been found for several other DNA-protein complexes (26), well separated and easily quantitated bands for DNA-protein complex and free DNA are seen in agarose gels with EcoRI binding. The stability of protein-DNA complexes during the gel experiment was verified using the technique described by Fried (27) and shown in Figure 2. Complexes of EcoRI and the 322 bp DNA fragment were loaded on agarose gels at 15 min intervals over a total time span of 2 hr. No dependence of the fraction of DNA in complex on the time of gel electrophoresis was observed.

Quantitation and data analysis

DNA bands on agarose gels were visualized and quantitated with ethidium bromide staining. The gels were photographed with a Panasonic BD 400 videocamera (averaging 128 frames) connected to Macintosh IIfx microcomputer, and using Foto/Prep model 3-3501 UV transilluminator (Fotodyne) for excitation of ethidium bromide fluorescence. Band intensities were measured using Image 1.45. The linearity of the system response was confirmed from the linearity of measured bands intensities vs. DNA size for pBR322 DNA fragments generated by MspI digestion. Raw gel data were always used for calculations. Computer-enhanced gel-pictures are shown in Figures to illustrate qualitative effects.

The nuclease protection experiments and the binding competition experiments using the gel mobility shift assay were analyzed as simple drug binding isotherms. If K is the equilibrium constant for drug-DNA complex formation, F_0 the fraction

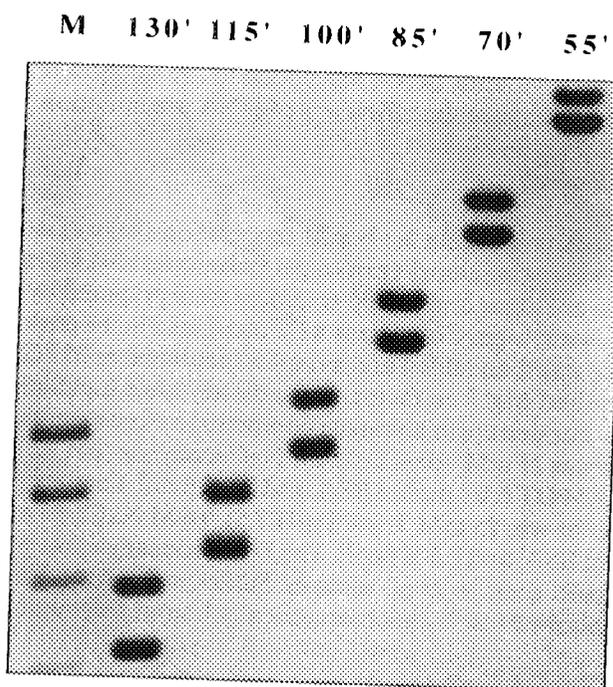


Figure 2: The EcoRI-DNA complex is stable during electrophoresis in agarose gels. A 322 bp fragment containing the EcoRI recognition site was incubated with sufficient EcoRI to bind about half the sites. Mixtures were loaded on a 1.5% agarose minigel at different times and electrophoresed at about 100 v. The fraction of EcoRI complexed DNA does not depend on the electrophoresis time. Lane M — pBR322. *MspI* markers (only the four biggest fragments — 622, 527, 404 and 307 bp — are seen on the gel). Lanes from left to right correspond to EcoRI-DNA mixtures run for the indicated times.

DNA not cleaved by enzyme or with no enzyme bound in the absence of drug, and F the fraction DNA uncut by enzyme or with no bound enzyme in the presence of a free concentration of netropsin or netropsin analogue $[Nt]_f$, then the protection from nuclease digestion or the decrease in EcoRI binding can be described by the standard normalized equations,

$$\frac{F - F_0}{1 - F_0} = \frac{K[Nt]_f}{1 + K[Nt]_f} \quad [1]$$

or

$$\frac{1 - F}{F - F_0} = \frac{1}{K[Nt]_f} \quad [2]$$

The data was fit to these equations using SigmaPlot 5.0 (Jandel Scientific Software).

The free netropsin and netropsin analogue concentrations ($[Nt]_f$) were taken as the total added drug concentrations. This assumption was verified by control experiments described above.

The expected protection from EcoRI cleavage by drug binding can be straightforwardly calculated assuming standard Michaelis-Menten enzyme kinetics (consistent with the observed dependence of fraction cleavage on enzyme concentration) and that bound drug prevents EcoRI binding. The computations show that the apparent drug binding constant determined from restriction nuclease protection is expected to depend somewhat on the initial extent of cleavage without added drug. For an initial 50% digestion, we calculate that the apparent binding constant will be about 40% smaller than the actual binding constant. The calculated protection curves, however, are surprisingly well fit by the simple binding equations given above.

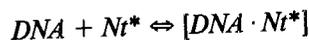
Under the conditions of the gel shift assay, binding of EcoRI is essentially stoichiometric without added drug, with a specific binding constant of about $10^{11} M^{-1}$ (28). It might seem unrealistic to expect netropsin to displace such a tightly bound protein except at very high concentrations. Straightforward calculations indicate that displaced specifically bound EcoRI will bind nonspecifically for the DNA concentrations used in the experiment. The ratio of specific and nonspecific binding constants for EcoRI is only about 10^4 (28). Our calculations show that the apparent drug binding constants determined from the loss of specific EcoRI binding will depend on the ratio of specific and nonspecific EcoRI binding constants and on the ratio of specific and nonspecific binding site concentrations. We calculate that for the experimental conditions used here the apparent drug binding constants extracted from the data could underestimate the real equilibrium constant by, at most, a factor of two. Once again, the simple binding equations given above very adequately fit the calculated data in spite of the complications inherent in the system.

Results

Netropsin analogue binding protects DNA from EcoRI nuclease digestion

The netropsin analogue (Nt^*) used here binds to DNA with a much lower affinity than netropsin itself (22). We previously measured the binding of this drug to chicken erythrocyte DNA using a traditional circular dichroism assay (29). The average binding constant in 0.1 M NaCl and at 25 °C was about $4 \times 10^5 M^{-1}$, about 50 fold smaller than for the binding of netropsin itself. Figure 3 shows the inhibition of EcoRI digestion of the DNA plasmid pUC19, linearized with Sca I, caused by analogue binding. DNA is completely protected from digestion by EcoRI with sufficiently high concentrations of analogue.

The protection observed in figure 3(a) can be described by a simple binding equilibrium,



where the complex, $[DNA \cdot Nt^*]$, is resistant to EcoRI digestion. A typical fit of the

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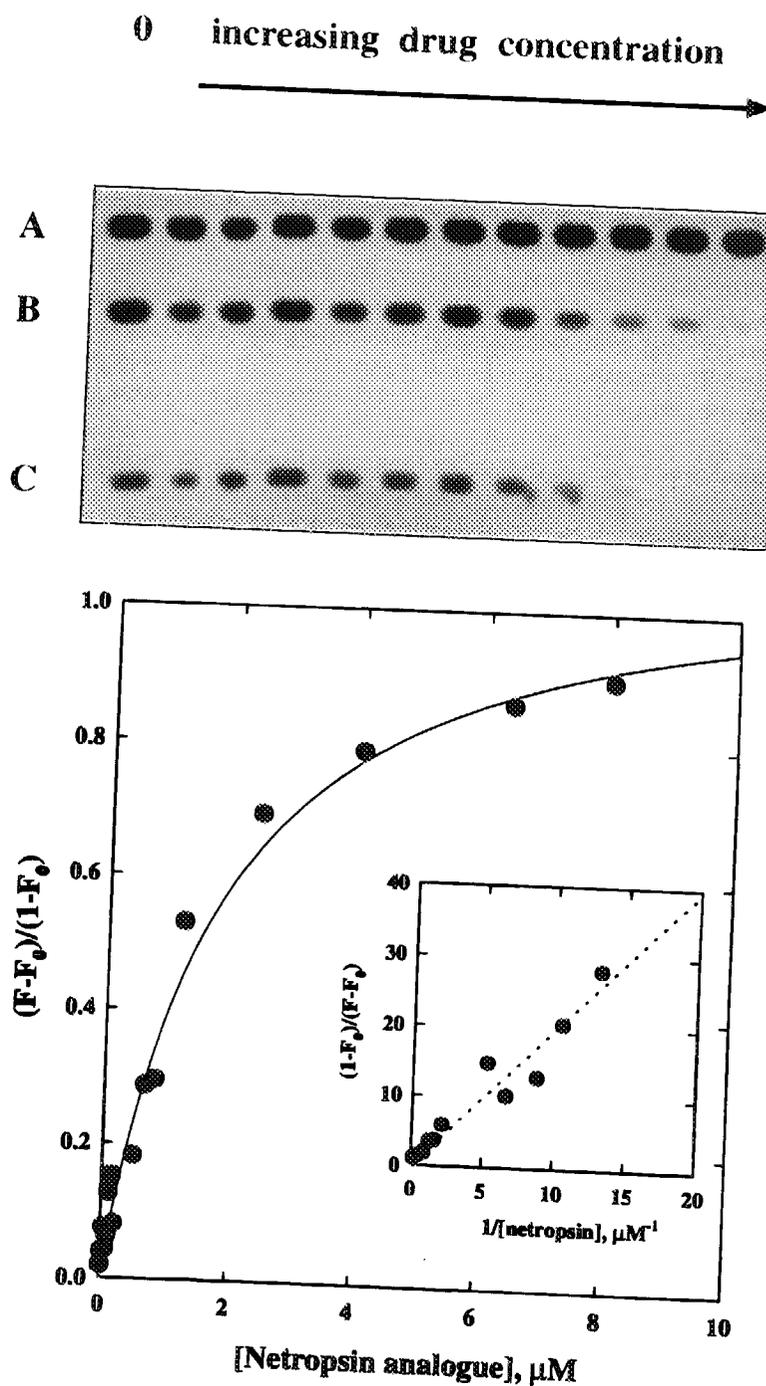


Figure 3 (a): Netropsin analogue protects the EcoRI site on pUC19 from digestion with EcoRI. Complexes of netropsin analogue and ScaI linearized pUC19 were prepared by direct titration of DNA solution with drug and incubated in the dark for 20 min on ice. EcoRI was then added and samples incubated
Figure Legend continued on next page.

at 25 °C for 30 min. After that reaction was stopped and DNA digested products were separated on 1% agarose gel. The positions of the undigested DNA (2686 bp long) and of the digestion products are given by A, B, and C, respectively. The leftmost lane is with no drug added. With increasing drug concentration a smaller fraction of DNA is cleaved. (b) The protection can be described by a simple binding reaction. The solid line is the best fit of the data (•) to equation [1]. F is the fraction uncleaved DNA and F_0 is the fraction uncleaved DNA in the absence of drug. In the inset, the linear version of the binding isotherm, equation [2], is shown.

drug titration data to this simple binding scheme is shown in Figure 3(b). The binding constant of the netropsin analogue to the EcoRI site of pUC19 in 0.1 M NaCl, 5 mM MgCl₂ and at 25 °C is $(8 \pm 1) \times 10^5 \text{ M}^{-1}$, determined by this restriction nuclease protection assay. This is about 2 fold higher than the average binding constant of the analogue to 'random sequence' DNA determined by circular dichroism (29). The difference is somewhat greater if the difference in ionic strength is accounted for, but still reasonable given the range of constants for the binding of netropsin to different dA/dT sequences (18).

Netropsin analogue binding competes with EcoRI binding

Netropsin analogue binding can protect DNA from EcoRI digestion in two possible ways: a direct competition for DNA binding or an inhibition of cleavage reaction subsequent to binding. The gel mobility shift assay can be used to quantitate the loss of EcoRI binding due to netropsin analogue competition. A typical gel-shift experiment in the presence of netropsin analogue is shown in Figure 4(a), using a 322 bp DNA fragment, containing the EcoRI site from pUC19. As the netropsin analogue concentration increases, the concentration of EcoRI-DNA complex decreases. At sufficiently high analogue concentrations, no complex is observed. A quantitative comparison of the nuclease protection and the binding competition titrations is shown in Figure 4(b). The protection from digestion is afforded at the level of EcoRI binding. As with nuclease protection, the competition can be analyzed with a simple netropsin analogue binding reaction, assuming the drug-DNA complex prevents EcoRI binding. The netropsin analogue binding constant calculated from gel-shift experiment is $(9.5 \pm 1.5) \times 10^5 \text{ M}^{-1}$, in good agreement with the $8 \times 10^5 \text{ M}^{-1}$ binding constant determined from protection, particularly if the difference in ionic strength is taken into account.

Netropsin — EcoRI competition.

Figure 5 shows nuclease protection data for the binding of netropsin itself to both the EcoRI (GAATTC) and the NdeI (CATATG) sites of pUC19. The binding of netropsin to the EcoRI site is clearly much stronger than binding of netropsin analogue. Control experiments confirm that the DNA concentration used was low enough such that the netropsin bound is a small fraction of the total netropsin added (10% maximum). Both sites are completely protected from digestion at sufficiently high drug concentrations. Analyzing the data as simple binding reaction, the apparent equilibrium constant for netropsin binding to the EcoRI site is $(2.5 \pm 0.5) \times 10^7 \text{ M}^{-1}$, about 30 fold higher than for the netropsin analogue. The apparent binding constant to the NdeI site is $(2.2 \pm 0.4) \times 10^6 \text{ M}^{-1}$, about 10 fold smaller than to the EcoRI site.

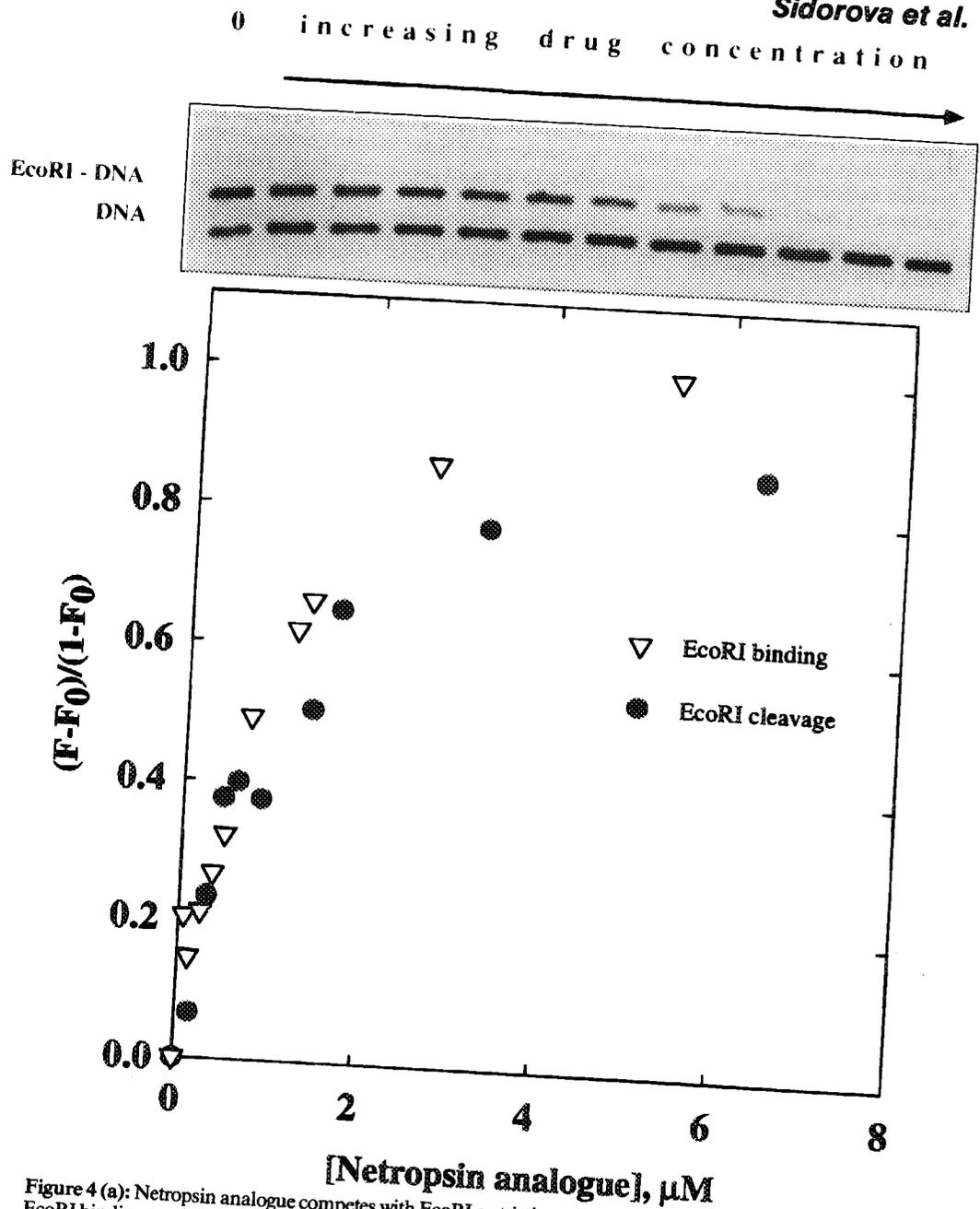


Figure 4 (a): Netropsin analogue competes with EcoRI restriction nuclease for DNA binding. The change in EcoRI binding to a 322 bp DNA fragment containing its recognition site is monitored by the gel mobility shift assay. Complexes of netropsin analogue and 322 bp fragment were first prepared by direct titration of DNA solutions with analogue. Complexes were incubated in the dark for 20 min on ice. EcoRI was then added and samples incubated at 25 °C for additional 30 min. The reaction mixture was then electrophoresed on a 1.5% agarose gel at 100 v for 1.5 hour. The leftmost lane is with no drug added. With increasing drug concentration, specific EcoRI binding decreases. (b) The loss of specific EcoRI binding with increasing netropsin analogue concentration is quantitatively comparable to the increase in EcoRI nuclease protection. The fraction uncomplexed DNA 322 bp fragment from the gel mobility shift experiment normalized as in equation [1] is shown as dependent on a drug concentration (▽). The normalized fraction of undigested 2686 bp DNA (from an experiment analogous to the one described in the legend to figure 3) is given by (●).

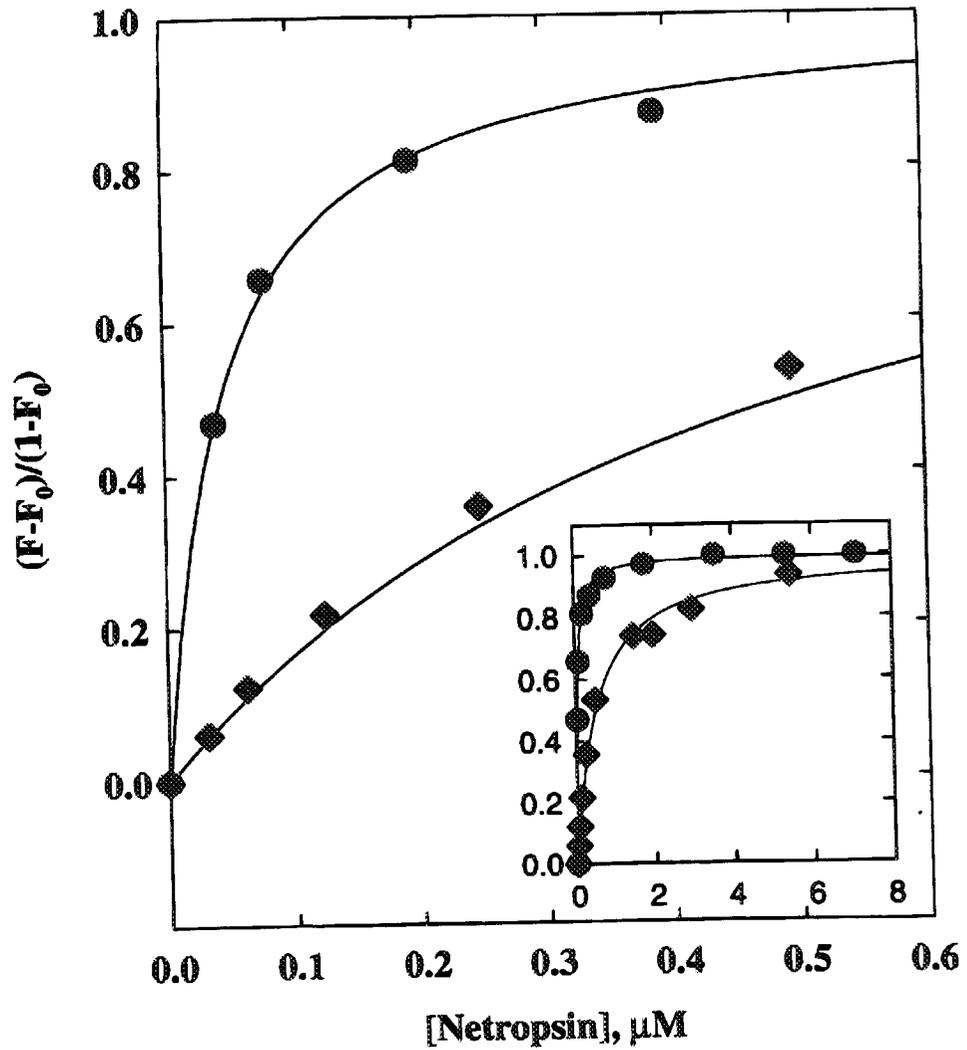
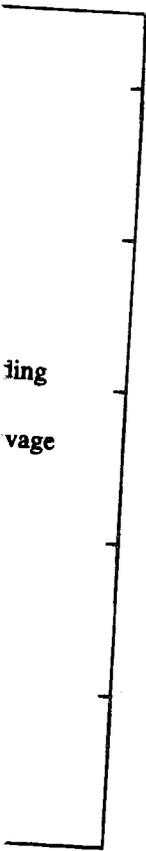
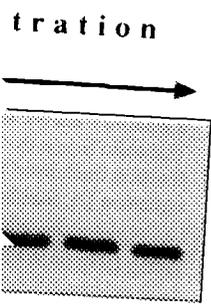


Figure 5: Netropsin binding to the EcoRI and NdeI sites of pUC19 determined by the nuclease protection assay. The experimental method is as described in *Methods and Materials* and in Figure 3. The fraction undigested 2686 bp DNA normalized for complete protection is given by (\bullet) for nuclease EcoRI (GAATTC) and by (\blacklozenge) for NdeI protection (CATATG). Solid lines are the best fit of the data to the equation [1] with binding constants $2.3 \times 10^7 \text{ M}^{-1}$ and $3.6 \times 10^6 \text{ M}^{-1}$ respectively. The figure inset shows protection at higher drug concentrations. Netropsin does fully protect the NdeI site.

That the nuclease protection results from netropsin DNA binding and not from a direct effect of the drug on the enzyme is shown in Figure 6. The protection from EcoRI cleavage afforded by netropsin binding (lanes 1 and 2) can be abolished by adding sufficient competitor DNA (lane 3). The PacI linker (CCTTAATTAAGG) used has at least one and possibly two strong binding sites for netropsin and can effectively compete with the EcoRI site for drug binding. The loss of protection at the EcoRI site is a consequence of the loss of drug binding at the EcoRI site due to competition. At the same time BamHI linker (CGCGGATCCGCG) could not effectively compete with the EcoRI site for the netropsin binding even at concentrations 8 times higher than the PacI linker concentration sufficient for complete

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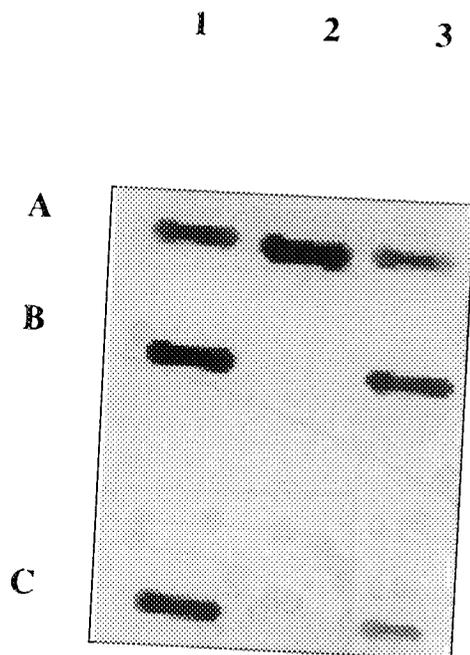


Figure 6: Nuclease protection due to drug binding is abolished by DNA competition for netropsin binding. In lane 1, cleavage of *Sca*I linearized pUC19 by *Eco*RI nuclease in the absence of netropsin is shown. The position of the undigested DNA is given by A; the digestion products by B and C. Lane 2: same conditions as lane 1, but with 0.1 μ M netropsin added to the reaction mixture. The DNA is protected from digestion by drug binding. Lane 3: same conditions as in lane 2 but with 0.9 μ M *Pac*I linker DNA also added. This oligonucleotide has a strong binding site for netropsin and competes with the *Eco*RI site. Even 0.2 μ M of *Pac*I linker is sufficient to decrease protection significantly.

loss of protection. This *Eco*RI assay of the competition for netropsin binding can, in fact, be used as an indirect method for measuring binding constants of netropsin to the competitor sequences (manuscript in preparation).

Netropsin binds too strongly to the *Eco*RI site at 0.1 M NaCl and no $MgCl_2$ to use the gel mobility shift assay and ethidium bromide staining to measure accurately the binding competition between *Eco*RI and drug. Binding competition can, however, be measured at 0.6 M NaCl. Figure 7(a) shows the loss of *Eco*RI-DNA complex with increasing netropsin concentration. Figure 7(b) shows the binding data and best fitting curve, assuming a simple competition. The netropsin binding constant at this salt concentration is about $(5.6 \pm 0.8) \times 10^6 M^{-1}$. Using the reported sensitivity of netropsin binding on salt concentration (30), this corresponds to a binding constant of about $6 \times 10^7 M^{-1}$ for the salt conditions used for nuclease protection. This is reasonably close to the constant derived from nuclease protection experiments, $2.5 \times 10^7 M^{-1}$.

Netropsin bound at EcoRI site protects neighboring sites from digestion

The *Eco*RI restriction sites with a central AATT sequence is expected to be a strong netropsin binding site. The sequence adjacent to the *Eco*RI site on pUC19, GAGCTC, is expected to bind netropsin only very weakly. Figure 8 shows, however, that the protection by netropsin of this sequence from nuclease digestion by either *Ban* II or *Sac* I is almost indistinguishable from the *Eco*RI site protection. The specific sequence recognized by *Ban* II or *Sac* I does not overlap with the GAATTC sequence of the *Eco*RI site. The *Kpn*I site (GGTACC) located another 6 bp from the *Eco*RI site is also expected to bind netropsin only very weakly. In this case, however, the restriction nuclease protection assay shown in Figure 8 now indeed shows no apparent binding of the drug and consequent protection over concentration range examined. The most reasonable explanation is that netropsin binding at the *Eco*RI site not

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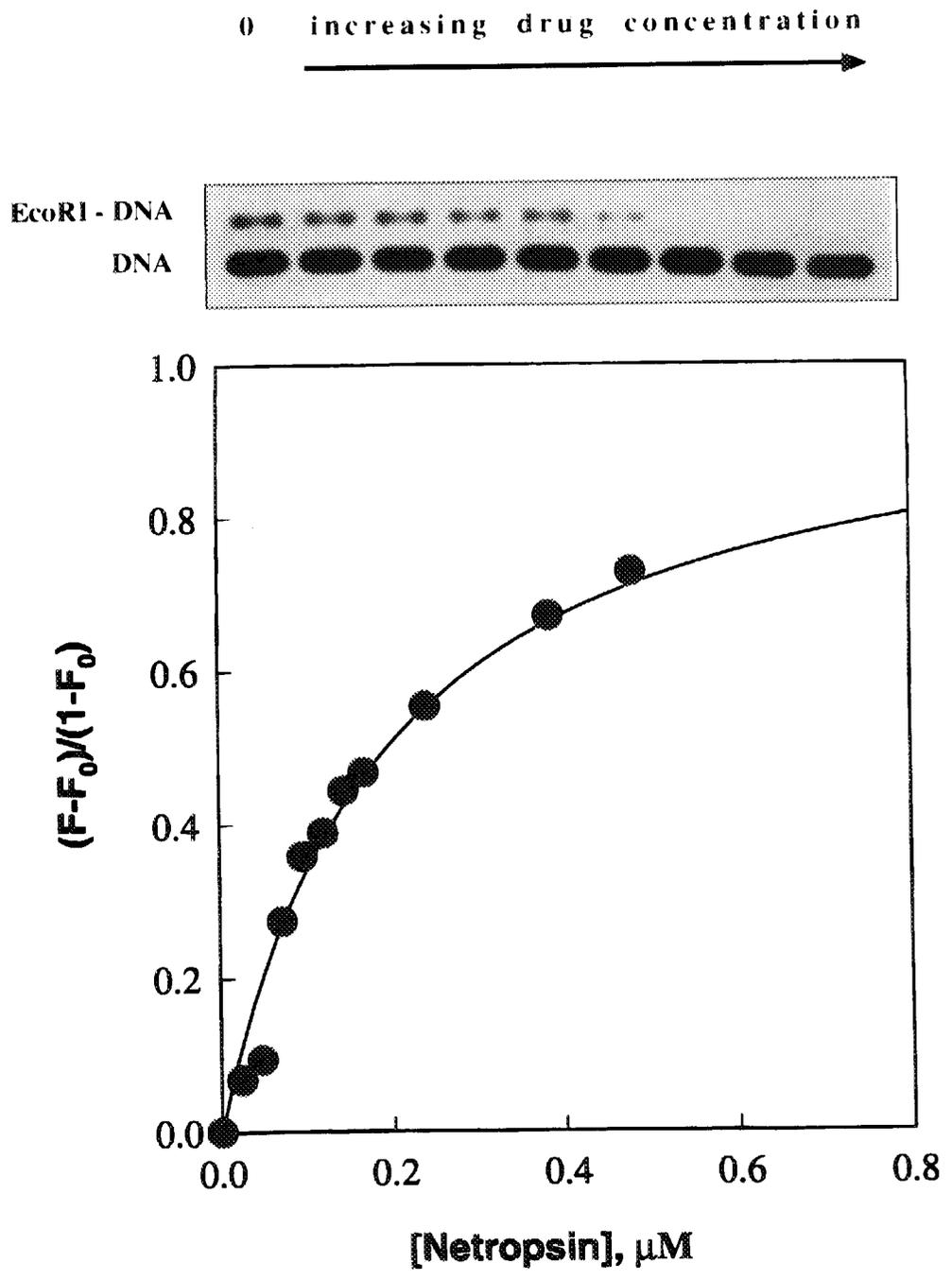


Figure 7: Netropsin competes with EcoRI nuclease for binding to DNA. Specific EcoRI binding to a 322 bp DNA fragment is monitored by the gel mobility shift assay. In (a), a typical gel is shown for the effect of increasing netropsin on the fraction of EcoRI-DNA complex. The leftmost lane is with no drug added. Complexes were formed at salt concentration of 0.675 M Na⁺ and 25 °C. In (b), the loss of specific EcoRI-DNA complex with increasing netropsin concentration is analyzed as a simple drug binding reaction. The solid line is the best fit of the data (•) to equation [1].

constants determined from direct binding assays. Our best estimates are that binding constants from the nuclease protection and the gel mobility shift assays may underestimate the actual constant by about 40 % and a factor of two, respectively.

Within the limitations of the experiment, the factor of two difference is not significant. As was shown in Figure 4b, protection from EcoRI nuclease digestion is coincident with the binding competition between netropsin analogue and EcoRI.

The binding of netropsin itself to the EcoRI site ($K_{b,corr} = 5.0 \times 10^7 M^{-1}$) by the nuclease protection assay is some 25 times stronger than binding of the analogue. The EcoRI/netropsin competitive binding constant is some 2-4 fold larger than the constant extracted from nuclease protection. The salt correction from 0.6 M NaCl to 0.1 M, however, is the longest extrapolation and most liable for error. Our previous results with netropsin analogue only showed a ratio of binding constants, determined by CD, between 0.1 and 0.3 M NaCl of 5.5. This corresponds to an exponent of only 1.55, instead of 1.6-1.8, and a calculated $K'_{b,corr} = 8.5 \times 10^7 M^{-1}$. Once again, we do not consider this factor of two difference in binding constants between different experimental methods as necessarily significant. As with the analogue, the results with netropsin are fully consistent with nuclease protection directly linked to binding competition.

The apparent binding constant of netropsin to the NdeI site (ATAT) determined by nuclease protection ($K_b \sim 2 \times 10^6 M^{-1}$) is about 10 fold weaker than to the EcoRI site (AATT). Both the magnitude of binding constants and the sensitivity to particular (dA/dT) sequences are consistent with previous measurements. Ward *et al.* (18), using DNase footprinting, showed that netropsin binding constants to different sites containing four consecutive dA/dT base pairs, could differ by more than 20 fold, depending on the specific sequence. The binding constants to sites with at least four consecutive dA/dT base pairs ranged from 0.2 to $16.0 \times 10^7 M^{-1}$ in 8 mM MgCl₂ and 2 mM CaCl₂ (approximately equivalent to 100 mM NaCl) depending on the specific sequence. Using the increase in DNA melting temperature accompanying netropsin binding and an enthalpy of binding, Marky *et al.* (34) measured a netropsin binding constant to a dodecamer containing the EcoRI recognition site as $2.8 \times 10^8 M^{-1}$, in 0.018 M Na⁺. The binding constant in 0.1 M NaCl is estimated as about $2 \times 10^7 M^{-1}$.

The protection of DNA from EcoRI cleavage by both netropsin and netropsin analogue is consistent with a competition between EcoRI and the drugs for binding to the recognition site. The binding of several other sequence specific DNA binding proteins has also been reported sensitive to competition from netropsin or distamycin, a closely related antibiotic with similar binding DNA characteristics (2). The specific binding of λ Cro protein to O_R3 (3), of OTF-1 and NFE (4), and of two homeodomain peptides, *Antp* HD and *ftz* HD, (5) to their respective recognition sequences are inhibited by netropsin or distamycin binding to dA/dT rich sequences. The inability of these proteins to bind in the major groove of DNA concurrent with drug binding in the minor groove could be due to a direct competition between protein and drug for interaction with the same DNA groups, i.e., these proteins

could make minor groove contacts that are important for binding stability. A critical role for direct protein-DNA minor groove contacts, however, was specifically discounted for *ftz* HD. A truncated peptide missing the N-terminal region that interacts with the minor groove is still strongly inhibited by drug binding (5).

Alternatively, the competition has also been attributed to distortions in DNA structure caused by drug binding that are large enough to preclude protein binding (4). An x-ray crystal structure of a netropsin-oligonucleotide complex (8, 9) shows, however, that at least with this drug only modest changes in the oligonucleotide structure are induced by binding. There is some widening of the minor groove (0.5-0.8 Å) and bending of the helix axis into the major groove by about 8°. In at least two cases, netropsin binding actually stabilizes the canonical B-form structure. The B-to A- form transition of calf thymus DNA induced by alcohol is strongly inhibited by both netropsin and distamycin binding (19, 35). In the triple helix, poly(dT) · poly(dA) · poly(dT), the second poly(dT) strand binds in the major groove of the double helix. Each chain in the triplex adopts an A-form like conformation (36). The transition temperature of the triple to double helix reaction, poly(dT) · poly(dA) · poly(dT) ⇌ poly(dA) · poly(dT) + poly(dT), decreases strongly with increasing netropsin concentration (20, 21). Netropsin binding in the minor groove essentially displaces a poly(dT) strand in the major groove (37).

The direct competition observed between netropsin and EcoRI only means that the binding of either drug or protein greatly weakens the concurrent binding of the other, i.e., that the structures are mutually incompatible. Equilibrium thermodynamics can not distinguish between a DNA structural change induced by drug binding that weakens protein binding from a protein binding induced change that interferes with drug binding. The decrease in binding constant of EcoRI to its recognition sequence caused by drug binding can be estimated from the limiting nuclease protection observed at high netropsin concentrations. Our data shows at least 95 % protection from nuclease digestion or EcoRI specific binding displacement from the gel mobility shift assay. This translates into a decrease in the binding constant of EcoRI to its specific recognition site of at least a factor of 20 when netropsin is also bound to the same site. The x-ray structures of the separate complexes suggest that the competition between drug and EcoRI for DNA binding very probably results from the distortion of DNA structure accompanying protein binding that is incompatible with drug binding. The change in DNA structure with bound EcoRI is much greater than with bound netropsin. Although the minor groove of the DNA dodecamer complexed with EcoRI is fully exposed to aqueous solution in the crystal (15-17), it is significantly distorted by the protein. The central six base pairs are underwound by some 25°, increasing both groove widths by some 3.5 Å. At the ends of the recognition sequence, DNA is bent into the minor groove by some 20° — 40° with a distortion of twisting angles extending 1-2 base pairs into the flanking sequences.

From this point of view, the observation by Oakley *et al.* (6) that GCN4, a leucine zipper motif DNA binding protein, and a distamycin analogue can bind in the major and minor grooves simultaneously to an overlapping DNA sequence is perhaps not particularly surprising. The DNA sequence examined was closely related to the AP-1 recognition sequence of GCN4, with a single base pair separating half-sites. The

crystal structure of the GCN4-AP-1 oligonucleotide complex [38] is remarkable for the lack of distortion of the DNA structure.

If restriction enzymes *BanII* and *SacI* distort the helical structure in sequences immediately adjacent to their recognition site, than binding of netropsin to the *EcoRI* site could account for the observed protection of the DNA cleavage with *BanII* and *SacI* (Figure 8). Netropsin protects the GAGCTC site directly adjacent to the *EcoRI* site on pUC19 with just about the same apparent binding constant as the *EcoRI* site. Netropsin would only very weakly bind directly to this site. Protection is most probably due to netropsin binding at the strong *EcoRI* AATT site. If *SacI* or *BanII* binding distorts the helix structure of the flanking sequences analogous to *EcoRI*, then concurrent binding of netropsin to the *EcoRI* site and of *SacI* or *BanII* can easily be mutually exclusive without a direct competition for minor groove contacts. The difference in nuclease protection between *BanII* and *SacI* at high netropsin concentrations indicates that *BanII*-netropsin competition is stronger than for *SacI*-netropsin. This suggests that DNA distortions in the flanking sequences due to *BanII* binding interfere with netropsin-DNA interactions more than with *SacI* binding.

Since, in general, helix distortions do not seem to propagate over more than a few base pairs (39, 40), it is not surprising that the *KpnI* site (GGTACC) located 6 base pairs away from *EcoRI* site on pUC19 is not protected by netropsin binding at the *EcoRI* site.

Conclusions

- The binding of netropsin or netropsin analogues can be easily and conveniently measured by a restriction nuclease protection assay.
- The nuclease protection afforded by drug binding is consistent with a binding competition between the drug and *EcoRI*.
- Since sequence specific DNA binding proteins often distort DNA structure, drugs meant to inhibit the action of these proteins need only stabilize the canonical structure of the recognition sequence. It is not necessary to design drugs to interact specifically with the same DNA groups as the protein.
- The extent of binding competition between netropsin and protein can perhaps be used as a qualitative indicator of DNA structure distortion caused by protein binding.

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