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Binding of Bis-linked Netropsin Derivatives in the Parallel-Stranded Hairpin Form to DNA

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Abstract

*C*is-diammine Pt(**II**)- bridged bis-netropsin and oligomethylene-bridged bis-netropsin in which two monomers are linked in a tail-to-tail manner bind to the DNA oligomer with the sequence 5′-CCTATATCC-3′ in a parallel-stranded hairpin form with a stoichiometry 1:1. The difference circular dichroism (CD) spectra characteristic of binding of these ligands in the hairpin form are similar. They differ from CD patterns obtained for binding to the same duplex of another bis-netropsin in which two netropsin moieties were linked in a head-to-tail manner. This reflects the fact that tail-to-tail and head-to-tail bis-netropsins use parallel and antiparallel side-by-side motifs, respectively, for binding to DNA in the hairpin forms. The binding affinity of cis -diammine Pt(**II**)- bridged bis-netropsin in the hairpin form to DNA oligomers with nucleotide sequences 5′-CCTATATCC-3′ (**I**), 5′-CCTTAATCC-3′ (**II**), 5′-CCTTATTCC-3′ (**III**), 5′-CCTTTTTCC-3′ (**IV**) and 5′-CCAATTTCC-3′ (**V**) decreases in the order **I** = **II** > **III** > **IV**> **V** . The binding of oligomethylene-bridged bis-netropsin in the hairpin form follows a similar hierarchy**.** An opposite order of sequence preferences is observed for partially bonded monodentate binding mode of the synthetic ligand.

Introduction

In the past decade a considerable progress has been achieved in the design and synthesis of compounds which can bind to DNA at selected sites. Most of the newly synthesized sequence-specific DNA-binding ligands are derivatives of antiviral and antitumor antibiotics netropsin and distamycin A (for reviews see refs. 1 and 2). X-ray (3-5) and NMR (6) studies show these two antibiotics bind in the minor DNA groove at runs of four or five AT- base pairs . Their binding specificity derives from specific hydrogen bonding interactions between the amide NH groups of the antibiotic molecule and the thymine O2 and adenine N3 atoms, van-der-Waals contacts with both sides of the minor groove and electrostatic interactions.

An obvious way to enhance the binding specificity shown by these antibiotics is to synthesize dimer compounds (bis-netropsins and bis-distamycins) in which two monomers are linked in head-to-head, head-to-tail and tail-to-tail orientations . Since the first communications on the design and DNA-binding properties of synthetic ligands of this class (7,8) a considerable progress has been achieved (9-21). It was shown that some of these compounds exhibit a high binding specificity and inhibit selectively initiation of transcription directed by certain procaryotic promoters (8). Bis-linked netropsin derivatives selectively inhibit activity of topoisomerases I and II (18,19), HIV-1 reverse transcriptase (20) and integrase (21).

It was shown that netropsin -peptide conjugates and bis-netropsins containing two-stranded antiparallel peptide motif can recognize DNA sites with mixed sequences of AT- and GC- base pairs (13,22). Recent NMR and X-ray studies of distamycin (lexitropsin)-DNA complexes have led to identification of a new DNA binding

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motif containing two distamycin (lexitropsin) molecules which are packed in an antiparallel side-by-side manner in the minor groove (23-29). The side-by-side antiparallel dimer motif was used by Dervan and coworkers for the elegant design of covalently linked polyamide dimers containing N-methylpyrrole- and N-methylimidazole-carboxamide units (14,31,32). These synthetic ligands in the hairpin form can recognize a broad category of nucleotide sequences on DNA (31- 33). They are cell permeable, and one of these compounds was shown to interfere with gene expression in cell cultures (34).

Recently it has been reported that two parallel oligopyrrolecarboxamide strands can be sandwiched in the minor DNA groove (35). Our previous studies show that *cis*-diammine Pt (**II**)-bridged bis-netropsin (←**Nt-Pt(NH_3)$_2$-Nt**→) (Figure 1) forms two types of complexes with poly[d(AT)]ï poly[d(AT)] and DNA oligomers containing sequences 5′-CC$(TA)_n$CC-3′, where n = 5 or 6 (34). The first type corresponds to the binding of ←**Nt-Pt(NH_3)$_2$-Nt**→ in the extended conformation and is characterized by the saturation ratio of one bis-netropsin molecule bound to approximately 8 or 9 successive AT-base pairs. The second type of the complex reflects binding of ←**Nt-Pt(NH_3)$_2$-Nt**→ in the hairpin form which contains two netropsin moieties in the parallel orientation.

Footprinting studies showed that ←**Nt-Pt(NH_3)$_2$-Nt**→ in the extended conformation binds selectively to DNA regions with sequences 5¢-TXTTAAYA-3¢ , where X and Y are predominantly T or A (36). However, little is known about the sequence preferences shown by tail-to-tail bis-netropsins in the parallel-stranded hairpin form. In the present work, we have studied the interaction of ←**Nt-Pt(NH_3)$_2$-Nt**→ and its analogue ←**Nt-(CH_2)$_5$-Nt**→ (Figure 1) with a series of 9-mer duplexes of the form 5′-CCXCC-3′ where X is TATAT (**I**), TTAAT (**II**), TTATT (**III**), TTTTT (**IV**) and AATTT (**V**). Each duplex contains a five base pair long AT-track to which bis-netropsin can bind in the hairpin and partially bonded monodentate binding modes. We have found that both ←**Nt-Pt(NH_3)$_2$-Nt**→ and ←**Nt-(CH_2)$_5$-NtÆ** bind to the DNA oligomer **I** with the central sequence TATAT in the hairpin form with a stoichiometry of 1:1. However, they form several types of complexes with DNA oligomers **II**, **III**, **IV** and **V**. Our preliminary NMR studies showed that ←**Nt-Pt(NH_3)$_2$-Nt**→ forms uniform 1:1 complexes with the nanomer and octamer duplexes with sequences 5′-CCTATATCC-3′ and 5′-CGTATACG-3′, most probably exclusively in the parallel-stranded hairpin form. Different modes of binding of the synthetic ligands to DNA can be discriminated by means of CD spectroscopy. The estimated contents of hairpin, dimer and monodentate binding modes can then be used to calculate Scatchard isotherms from which thermodynamic parameters corresponding to different ligand binding modes can be determined by nonlinear least square fitting of the experimental and calculated binding curves.

Materials and Methods

Ligands

Figure 1 shows the chemical structures of netropsin **(Nt**), distamycin (**Dst**) and *cis*-diammineplatinum (**II**)-bridged bis-netropsin **(←Nt-Pt(NH_3)$_2$- Nt→),** oligomethylene-bridged bis-netropsin **(←Nt-(CH_2)$_5$-Nt→)** and triglycine-bridged bis-netropsin. Each synthetic ligand is composed of N-propylpyrrole residues which are replaced for N-methylpyrrole residues of the parent antibiotic netropsin. **(←Nt→ (Gly)$_3$-Nt→)**. ←**Nt-Pt(NH_3)$_2$-Nt**→, ←**Nt-(CH_2)$_5$-Nt**→ and ←**Nt→(Gly)$_3$-Nt**→ were synthesized and purified as described elsewhere (37). The concentrations of each bis-netropsin was determined spectrophotometrically, using the molar extinction coefficient at 297 nm of 42,000 M^{-1} cm^{-1}. Spectrophotometric measurements were carried out on a Cary1E instrument. The CD spectra were recorded on a Jasco Model 715 instrument, using 0.1, 0.2 and 1.0 cm pathlength cells. Unless specified, all measurements were carried out at 20°C in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl.

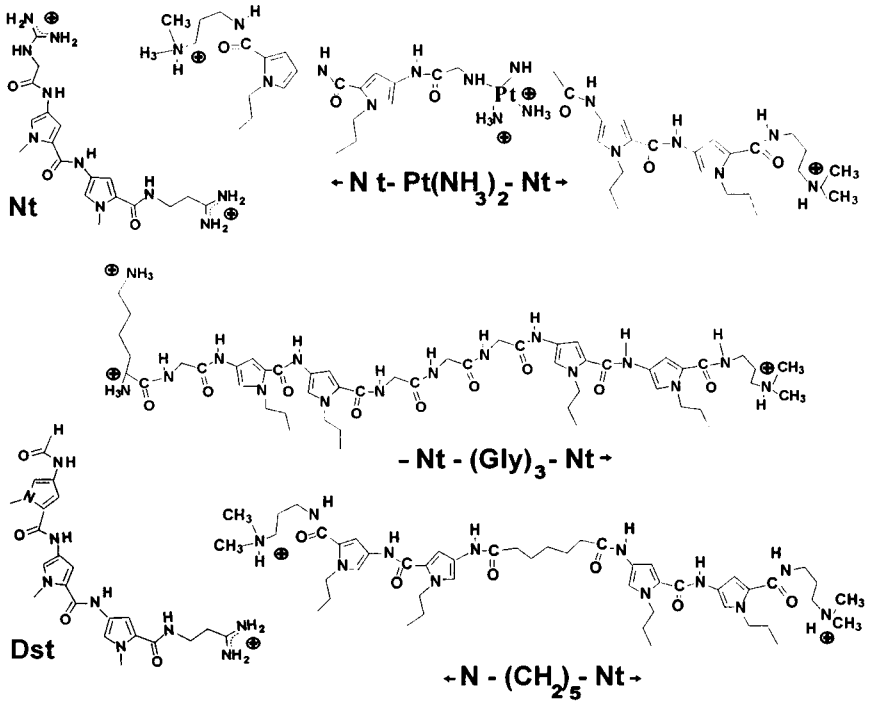

Figure 1: Chemical structure of netropsin (**Nt**), distamycin (**Dst**), *cis*-diammine Pt(II)-bridged bis-netropsin (←**Nt-Pt(NH$_3$)$_2$-Nt**→), oligomethylene-bridged bis-netropsin (←**Nt-(CH$_2$)$_5$-Nt**→) and triglycine-bridged bis-netropsin (-**Nt-(Gly)$_3$-Nt**→). The arrows indicate direction from the N-terminus to the C-terminus in each peptide fragment.

DNA Fragments

Oligonucleotides were synthesized on the basis of phosphoramidite chemistry by using of an automated synthesizer from Applied Biosystems. The following oligonucleotides were used in the present study:

5′-CCTATATCC-3′ (1a)	5′-GGATATAGG-3′ (1b)
5′-CCTTAATCC-3′ (2a)	5′-GGAATTAGG-3′ (2b)
5′-CCTTATTCC-3′ (3a)	5′-GGAATAAGG-3′ (3b)
5′-CCTTTTTCC-3′ (4a)	5′-GGAAAAAGG-3′ (4b)
5′-CCAATTTCC-3′ (5a)	5′-GGTTAAAGG-3′ (5b)

Concentrations of the oligonucleotides were estimated by measuring optical densities at 90°C, using the molar extinction coefficients at 260 nm of 88100 $M^{-1}cm^{-1}$ for oligonucleotides 1a, 3a, 5a; 111700 $M^{-1}cm^{-1}$ for oligonucleotides 1b, 3b, 5b; 82100 $M^{-1}cm^{-1}$ and 117700 M^{-1} for oligonucleotides 2a and 2b, respectively; 76100 $M^{-1}cm^{-1}$ and 84200 $M^{-1}cm^{-1}$ for oligonucleotides 4a and 4b, respectively. Annealing of complementary strands to form duplexes **I** to **V** containing oligonucleotides 1a+1b (**I**), 2a+2b (**II**), 3a+3b (**III**), 4a+4b (**IV**) and 5a+5b (**V**) was accomplished by heating the solutions containing complementary oligonucleotides in equimolar quantities and then cooling over night to the room temperature. The melting curves obtained for duplexes **I**, **II**, **III**, **IV** and **V** exhibit only one transition of the double-helical DNA oligomer.

Decomposition of Difference CD Spectra for Bis-Netropsin-DNA Complexes and Determination of Thermodynamic Parameters

Let R_1, R_2 and R_3 be the occupancy of the DNA oligomer by bis-netropsin bound in the monodentate mode, hairpin and selfassociated dimer forms, respectively. It is assumed that each DNA oligomer contains only one binding site for the ligand. The difference CD spectra for bis-netropsin-DNA complexes can be represented as a superposition of three reference CD patterns corresponding to the monodentate bis-netropsin binding, interaction in the parallel-stranded hairpin, and binding in the antiparallel dimer forms, respectively.

$$(\Delta D - \Delta D_0)/O = R_1\Delta\varepsilon_1(\lambda) + R_2\Delta\varepsilon_2(\lambda) + R_3\Delta\varepsilon_3(\lambda) \quad [1]$$

$$R_1 = K_1 m/Z \quad [2]$$

$$R_2 = K_2 m/Z \quad [3]$$

$$R_3 = 2Wm2/Z \quad [4]$$

$$m = C - O(R_1 + R_2 + R_3) \quad [5]$$

$$W = K_d K_3 \quad [6]$$

$$Z = 1 + K_1 m + K_2 m + Wm^2 \quad [7]$$

where C is the concentration of the ligand. Z is the grand partition function for the system under study. The partition function Z is equal to the sum of the statistical weights describing formation of different complexes between bis-netropsin and DNA oligomer. The statistical weighting factor for the state when DNA oligomer contains no bound ligand is assumed to be equal to 1. The numerical coefficient in the second term in Eq. [7] is a degeneracy factor inherent to the states corresponding to monodentate bis-netropsin binding. K_1 and K_2 are the affinity constants for monodentate bis-netropsin binding and interaction in the hairpin form, respectively. K_d is the dimerization constant. K_3 is the binding constant of dimer species to the DNA oligomer. ΔD is the measured CD amplitude of bis-netropsin-DNA oligomer mixture at wavelength λ expressed per mole of the DNA oligomer and 1 cm pathlength cell. ΔD_0 is the same quantity obtained for the naked DNA oligomer. O is the molar concentration of the DNA oligomer. C is the ligand concentration in the titration assay. m is the concentration of the free ligand. $\Delta\varepsilon_1(\lambda)$ and $\Delta\varepsilon_2(\lambda)$ are the molar dichroism values at wavelength λ corresponding to monodentate bis-netropsin binding and binding in the hairpin form, respectively. $\Delta\varepsilon_3(\lambda)$ is the molar dichroism value characteristic of antiparallel 2:1 complex between bis-netropsin and DNA oligomer.

Decomposition of the difference CD spectra into its constituents was achieved by least square fitting of the experimental and calculated CD contours, using the reference CD spectral profiles for binding of bis-netropsin in the extended conformation, hairpin and self-associated dimer forms. Decomposed CD spectral profiles obtained at different molar ratios of ligand to the DNA oligomer can be used for the assignment and quantitation of different bound ligand species present in the system under study. The affinity constants for monodentate binding and interaction in the hairpin form were determined from intercepts of individual binding isotherms in Scatchard representation on the vertical axis.

$$\lim_{R_1 \to 0} R_1 / m = K_1 \quad [8]$$

$$\lim_{R_2 \to 0} R_2 / m = K_2 \quad [9]$$

Here K_1 and K_2 are the equilibrium association constants for partially bonded monodentate binding and interaction in the hairpin form, respectively.

Results

Different Modes of Binding of Tail-to-Tail and Head-to-Tail Bis-netropsins to Poly[d(AT)] Poly[d(AT)] and DNA Oligomers

Figure 2 displays the CD spectra of complexes between ←**Nt-Pt(NH$_3$)$_2$-Nt**→ and poly[d(AT)]• poly[d(AT)] recorded at relatively low molar ratios of ligand to DNA base pairs (C/BP<0.1). Here C is the concentration of the ligand. BP is the molar concentration of the DNA base pairs. The maximum of the induced CD band occurs at 314 nm. The CD spectra exhibit an isodichroic point at 290 nm. The CD spectra

are similar to those observed for complexes of ←**Nt-Pt(NH_3)$_2$-Nt**→ with poly(dA) •poly (dT). The CD absorbance at 310 nm per mole of bound ligand (85 ± 5) is found to be approximately twice that found for binding of netropsin to poly(dA)• poly(dT) (13). This indicates that at low extents of binding ←**Nt-Pt(NH_3)$_2$-Nt**→ binds to poly[d(AT)]•poly[d(AT)] in the extended conformation in such a way that both monomer fragments of the bis-netropsin molecule are bound to AT-base pairs. Each bound ligand occupies approximately 8 or 9 successive AT -base pairs. Similar CD spectral profiles were recorded for complexes of ←**Nt-(CH_2)$_5$-Nt**→ and **-Nt→(Gly)$_3$-Nt**→ with poly[d(AT)] •poly[d(AT)] at low C/BP values. Increasing the C/BP value from 0.1 to 0.2 leads to considerable changes in the CD spectral profiles which reflect formation of complexes of the second type between ←**Nt-Pt(NH_3)$_2$-Nt**→ and DNA. Our previous studies shows that in systems containing short DNA oligomers of the form 5′-CC(TA)n CC-3′ (n=5, 6) the equilibrium between complexes of the first and second type is shifted toward the formation of complex of the second type (35).

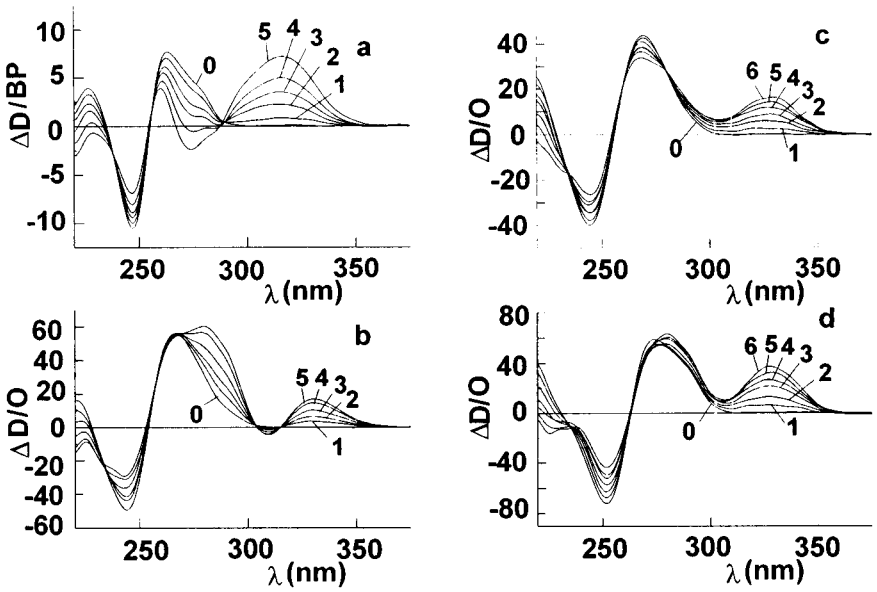

Figure 2: *(a),* CD spectra of complexes between ←**Nt-Pt(NH_3)$_2$-Nt**→ and poly[d(AT) poly[d(AT)] (45 μM base pairs) at different molar ratios of the ligand to DNA base pairs (C/BP). The C/BP values were as follows:, 0 (0), 1 (0.01), 2 (0.02), 3 (0.04), 4 (0.06), 5 (0.08). ΔD is the measured CD amplitude at a given wavelength calculated per 1 cm pathlength cell. *(b),* CD spectra of complexes between ←**Nt-Pt (NH_3)$_2$-Nt**→ and DNA oligomer with the sequence 5′-CCTATATCC-3′ (61 mM) at different molar ratios of ←**Nt-Pt (NH_3)$_2$-Nt**→ to the duplex (C/O). The C/O values were as follows: 0, 0; 1, 0.09; 2, 0.27; 3, 0.55; 4, 0.91; 5, 1.37; 6, 1.91. *(c),* CD spectra of complexes between **-Nt-(Gly)$_3$-Nt**→ and 9-mer duplex with the sequence 5′-CCTATATCC-3′ (109 μM). The C/O values were as follows: 0, 0; 1, 0.15; 2, 0.40; 3, 0.59; 4, 0.99; 5, 1.48; 6, 2.47. The CD spectra were recorded in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.01 M NaCl at 20°C. *(d),* CD spectra of complexes between **-Nt-(Gly)$_3$-Nt**→ and decamer duplex with the sequence 5′-GCTATATAGC-3′ (56.5 μM). The C/O values were as follows: 0, 0; 1, 0.49; 2, 0.97; 3, 1.46; 4, 2.92; 5, 4.9; 6, 7.8. The CD spectra were recorded in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.01 M NaCl at 20°C.

Figure 2 shows the CD spectra for complexes between ←**Nt-Pt(NH_3)- Nt**→ and 9-mer duplex with the sequence of 5′- CCTATATCC - 3′. The CD spectra exhibit two positive CD bands at 290 nm and 330 nm, with a negative CD contribution at 310 nm (Figure 2, *b*). The maintenance of a well-defined isodichroic point at 303 nm indicates that there is only one bound bis-netropsin species in the titration assay. From position of breakpoints on the titration curves measured at 310 nm and 340 nm one can conclude that binding approaches saturation when one bis-netropsin molecule is bound to the 9-mer duplex, irrespective of whether titrations were carried at 310 nm or 340 nm (Figure 3, panel *a*). These CD spectral profiles reflect binding of Pt-bridged bis-netropsin in the parallel-stranded hairpin form.

Distinctly different CD spectral profiles are observed on binding to the same 9-mer duplex of another bis-netropsin in which two netropsin-like fragments are linked in a head-to-tail manner via a tripeptide Gly-Gly-Gly (**-NtÆ(Gly)$_3$-Nt**→). The CD spectra exhibit a positive CD band at 328 nm (Figure 2, panel *c*). The isodichroic points are observed at 281 nm, 260 nm and 234 nm. This suggests that only one type of the complex is formed between the ligand and 9-mer duplex under these conditions. The binding approaches saturation level when one bis-netropsin molecule is bound to the 9-mer duplex (Figure 3, panel *b*). We propose that these CD spectral profiles reflect binding of **-NtÆ(Gly)$_3$-Nt**→ in the hairpin form which is built on the basis of antiparallel side-by-side motif.

The CD spectral profiles recorded for complexes between **-NtÆ(Gly)$_3$-Nt**→ and

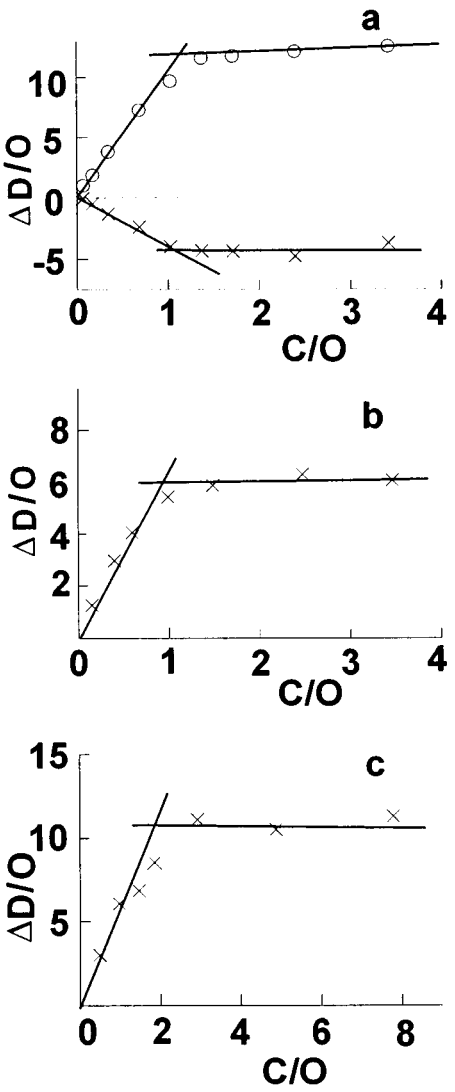

Figure 3: CD titrations of the 9-mer duplex with the sequence 5′-CCTATATCC-3′ (10.6 μM) by ← **Nt-Pt(NH$_3$)$_2$-Nt**→ at 310 nm (×)and 340 nm (○) (panel *a*) and CD titrations at 310 nm (×) of the same 9-mer duplex (109 μM) (panel *b*) and 10-mer duplex with the sequence 5′-CGTATATACG-3′ (56.5 μM) → by **-Nt-(Gly)$_3$-Nt**→ (panel *c*). CD titrations were carried out either in 1mM sodium cacodylate buffer (pH 7) in the presence of 0.1 M NaCl (panel *a*) or in the same buffer in the presence of 0.01 M NaCl (panels *b* and *c*).

10-mer duplex with the sequence 5′- GCTATATAGC - 3′ at low C/O values (C/O < 1) are similar to those obtained for complexes of **-Nt→ (Gly)$_3$-Nt**→ with the 9-mer duplex (Figure 2, panel *d*). Increasing the C/O value is accompanied by spectral alterations which reflect formation of a complex of the second type between **-Nt→ (Gly)$_3$-Nt**→ and DNA decamer. The binding approaches saturation when two bis-netropsin molecules are bound to the decamer duplex (Figure 3, panel *c*). We interpret these observations as indicating that complex of the first type containing bis-netropsin in the hairpin-like conformation is replaced at high C/O values by complex of the second type which is built on the basis of intermolecular side-by-side dimer motif similar to that observed in the crystalline complex between distamycin and inosine-containing octamer duplex d(IC)$_4$ (28). Molecular model building studies show that two netropsin moieties, one from each bound bis-netropsin molecule, can be packed in an antiparallel side-by-side fashion in the minor DNA groove of the central AT-track, with the other two netropsin-like fragments being in an unbound state.

The observed CD spectral alterations can be more clearly seen if presented as difference CD spectral profiles where contributions of the nucleic acid component are subtracted from the CD spectra of the corresponding complexes with the synthetic ligand. The results are shown in Figure 4 for complexes of synthetic ligands with poly[d(AT)]•poly[d(AT)] and DNA oligomers with sequences 5′- CCTATATCC - 3′ and 5′- GCTATATAGC - 3′. The CD absorbance at a given wavelength was calculated per 1 cm pathlength cell and divided on the molar concentration of bound bis-netropsin. Remarkably, the difference CD spectra reflecting the binding of ←**Nt-(CH$_2$)$_5$-Nt**→ and ←**Nt-Pt(NH$_3$)-Nt**→ to the same 9-mer duplex are similar (see Figures 5 and 6, panels *a*). They differ from the CD difference spectral profiles observed for binding to the same 9-mer duplex of **-Nt→(Gly)$_3$-Nt**→ which contains two netropsin-like fragments linked in a head-to-tail manner.

DNA Sequence Preferences for Binding of ←Nt-Pt(NH$_3$)-Nt→ and ←Nt-(CH$_2$)$_5$-Nt→ in the Parallel-Stranded Hairpin Form

In order to elucidate the possible sequence specificity on binding of ←**Nt-Pt(NH$_3$)-Nt**→ and ←**Nt-(CH$_2$)$_5$-Nt**→ in the hairpin forms we have studied their interaction with a series of 9-mer duplexes of the form 5′- CCXCC - 3′ where X=TATAT (**I**), TTAAT (**II**), TTATT (**III**), TTTTT (**IV**) and AATTT (**V**). Representative difference CD spectra for complexes between these synthetic ligands and DNA oligomers are shown in Figures 5 and 6 where sequence-dependent spectral differences between different complexes are evident.

In contrast to the results reported above for complexes of ←**Nt-Pt(NH$_3$)- Nt**→ with the DNA oligomer **I**, the CD patterns for complexes of ←**Nt-Pt(NH$_3$)- Nt**→ with the DNA oligomer **V** exhibit a single positive CD band with a peak near 315 nm. This type of CD pattern is reminiscent of bis-netropsin binding to poly(dA)• poly(dT) and poly[d(AT)]• poly[d(AT)] in the extended conformation. However, the molar CD absorbance at 310 nm is found to be approximately two times lower, thereby indicating that only one of the two netropsin-like fragments of the bis-netropsin molecule is bound to DNA oligomer **V**, with the other netropsin-like fragment being free (monodentate binding).

The CD spectral profiles obtained for complexes of ←**Nt-Pt(NH$_3$)- Nt**→ and ←**Nt-(CH$_2$)$_5$-Nt**→ with DNA oligomers **II**, **III**, **IV** and **V** can be well represented as a superposition of two (←**Nt-Pt(NH$_3$)- Nt**→) or three (←**Nt-(CH$_2$)$_5$-Nt**→) reference CD patterns: the difference CD pattern corresponding to the monodentate bis-netropsin binding and the CD spectra reflecting the interaction of each synthetic ligand in the parallel-stranded hairpin and self-associated dimer forms. The reference CD pattern characteristic of binding in the hairpin form can be calculated from the CD spectra recorded for complexes between each synthetic ligand and 9-

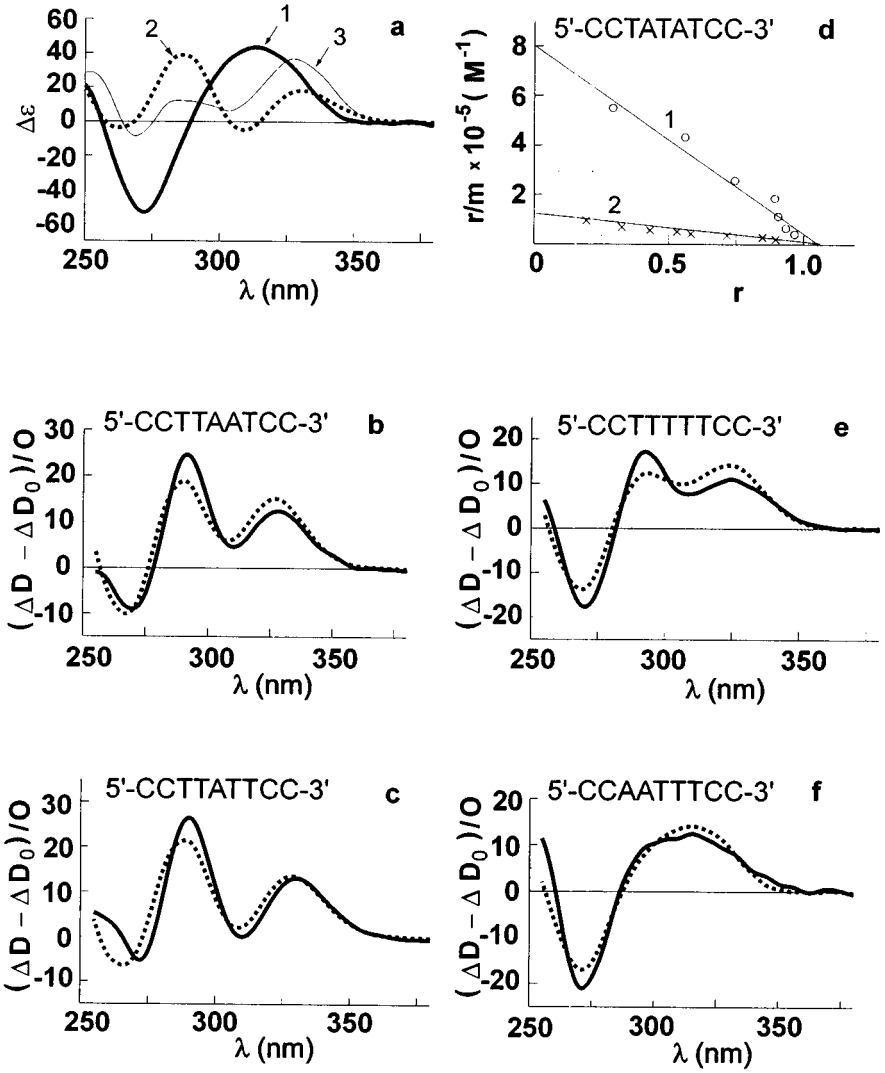

Figure 4: CD difference spectra characteristic of bis-netropsin binding in the extended conformation (*a*), hairpin form with parallel (*b*) or antiparallel (*c*) orientation of two netropsin moieties and antiparallel dimer (*d*) form. Panel *a*, CD difference spectrum obtained by subtracting the CD spectrum of poly[d(AT)]•poly[d(AT)] (44.7 μM, base pairs) from the CD spectrum of the complex formed by ←**Nt-Pt(NH$_3$)$_2$-Nt**→ with poly[d(AT)]•poly[d(AT)] at C/BP=0.038). Panel *b*, CD difference spectrum obtained by subtracting the CD spectrum of the 9-mer duplex 5′-CCTATATCC-3′ (56.6 μM) from the CD spectrum of the complex formed by ←**Nt-Pt(NH$_3$)$_2$-Nt**→ with the 9-mer duplex at C/O =1.56. Panel *c*, CD difference spectrum obtained by subtraction of the CD spectrum of the 9-mer duplex with the sequence 5′-CCTATATCC-3′ (73.0 μM) from the CD spectrum of the complex formed by -**Nt-(Gly)$_3$-Nt**→ with the 9-mer duplex at C/O=2.47. CD difference spectrum obtained by subtracting the CD spectrum of the 10-mer duplex 5′-GCTATATAGC-3′ (31.4 μM) from the CD spectrum of the complex formed by -**Nt-(Gly)$_3$-Nt**→ with the decamer duplex at C/O=7.8. The CD amplitudes in the difference CD patterns were divided on the concentration of bound bis-netropsin in the titration assays. Illustrated are the general plans of the complex structures formed by ←**Nt-Pt(NH$_3$)$_2$-Nt**→ and -**Nt**→**Gly$_3$-Nt**→ in the extended conformation, hairpin and dimer forms, respectively. The hairpins are built on the basis of parallel and antiparallel side-by-side peptide motifs, respectively.

mer duplex with the sequence 5′- CCTATATCC - 3′, whereas the CD pattern corresponding to the monodentate binding is reminiscent to that found for binding of Pt-bridged bis-netropsin in the extended conformation to poly [d(AT)]• poly[d(AT)]. However, the CD intensities should be divided by a factor 2, since only one netropsin-like fragment of the bis-netropsin molecule is bound to DNA. The CD spectra obtained for complexes between -**Nt**→**(Gly)$_3$-Nt**→ and decamer duplex with the sequence 5′- GCTATATAGC - 3′ at ligand/DNA oligomer molar ratios approaching the saturation level of binding were used to calculate the CD pattern corresponding to complexes which contain antiparallel side-by-side dimer motif. As before, the intensities of CD bands were divided on the molar concentration of bound ligand in the titration assay.

Figures 5 and 6 show that there is a good fit of the experimental and calculated CD spectral profiles in the wavelength region of 255 -380 nm. Decomposition of the difference CD spectra into components corresponding to different ligand binding modes allows assignment and quantization of bound ligand species at different C/O values. The affinity constants for monodentate binding and interaction in the hairpin form can be found from the intercepts on the vertical axis of the initial linear parts of binding isotherms in Scatchard representation (see Eqs. [8] and [9]).

Figure 5 (panel *d*) shows typical Scatchard isotherms obtained from binding in the hairpin form of Pt- and oligomethylene-bridged bis-netropsins to the 9-mer duplex with the sequence 5′- CCTATATCC - 3′. Examination of Scatchard plots shows that both synthetic ligands bind to the 9-mer duplex with a stoichiometry 1:1. The bind-

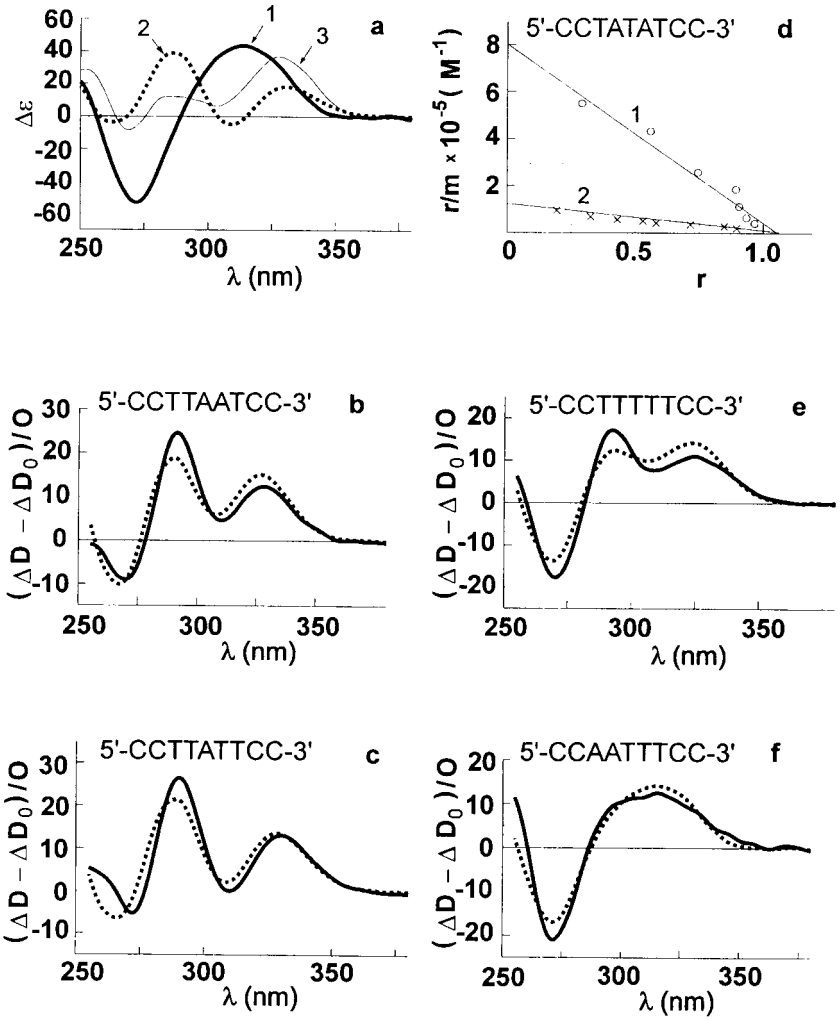

Figure 5: Decomposition of difference CD patterns for complexes of ←**Nt-Pt(NH_3)$_2$-Nt**→ with DNA oligomers **II** (20.4 μM, panel *b*), **III** (21.9 μM, panel *c*), **IV** (19.7 μM, panel *e*) and **V** (17.6 μM, panel *f*) into components which correspond to monodentate bis-netropsin binding and interaction in the hairpin and self-associated dimer forms. Panel *a*, difference CD patterns characteristic of monodentate binding of ←**Nt-Pt(NH_3)$_2$-Nt**→ (1) and binding in the hairpin (2) and dimer (3) forms. The C/O values for complexes of ←**Nt-Pt(NH_3)$_2$-Nt**→ with DNA oligomers **II**, **III**, **IV** and **V** were 2.06, 1.86, 2.55 and 1.96, respectively. Indicated are the nucleotide sequences for DNA oligomers **II, III, IV** and **V** The experimental and calculated difference CD patterns are shown by solid and dotted lines, respectively. The best fit between the experimental and calculated CD patterns is observed when the occupancy of the DNA oligomer by ←**Nt-Pt(NH_3)$_2$-Nt**→ binding in the monodentate and hairpin modes were as follows: panel *b*, R_1= 0.203 ± 0.010 , R_2 = 0.496 ± 0.018; panel *c*, R_1= 0.117 ± 0.008, R_2= 0.577 ± 0.015; Panel *e*, R_1=0.277 ± 0.008; R_2=0.288 ± 0.014; panel *f*, R_1= 0.324 ± 0.008; R_2= 0.053 ± 0.014. Panel *d*, Scatchard isotherms for binding of ←**Nt-Pt (NH_3)$_2$-Nt**→ (○) and (←**Nt-(CH_2)$_5$-Nt**→) (×) in the hairpin form to the 9-mer duplex with the sequence 5′-CCTATATCC-3′.

ing is characterized by association constants of 6.8 10^5 M^{-1} and 1.3 10^5 M^{-1} for ←**Nt-Pt(NH_3)- Nt**→ and ←**Nt-(CH_2)$_5$-Nt**→, respectively. The affinity constants for monodentate binding of ←**Nt-Pt(NH_3)- Nt**→ and ←**Nt-(CH_2)$_5$-Nt**→ to duplexes **I**, **II**, **III**, **IV** and **V** and their binding in the hairpin form are listed in Table I.

Measured ligand-induced changes in the thermal stability of the duplex **I** correlate well with these estimates of binding constants of Pt- and oligomethylene- bridged bis-netropsins to the duplex (Figure 7). The observed differences in the melting temperatures of the ligand free and ligand-saturated duplexes (ΔT_m) are equal to 18±1 and (6±0.5) °C for complexes of ←**Nt-Pt(NH_3)- Nt**→ and ←**Nt-(CH_2)$_5$-Nt**→ with duplex **I** (58 μM) at C/O values of 1.1 and 1.3, respectively. The polyamide-induced changes in duplex thermal stability reflect the fact that each bis-netropsin exhibits a preference for binding to duplex relative to its interaction with corresponding single-stranded oligonucleotides.

Table I

Binding Constants of ←**Nt-Pt(NH_3)$_2$-Nt**→ and ←**Nt-(CH_2)$_5$-Nt**→ to Various DNA oligomers.

	Nt-Pt(NH_3)$_2$·Nt		**Nt-(CH_2)$_5$-Nt**	
DNA oligomer	K_1, M^{-1}	K_2, M^{-1}	K_1, $\cdot M^{-1}$	K_2, M^{-1}
5′-CCTATATCC-3′	Not determined	(6.8±0.6) x 10^5	(3.9±0.8) x 10^3	(1.3±0.2) x 10^5
5′-CCTTATTCC-3′	(6.8±0.7) x 10^4	(2.3±0.4) x 10^5	(6.8±0.5) x 10^3	(1.4±0.2) x 10^4
5′-CCTTAATCC-3′	(3.8±0.7) x 10^5	(6.8±0.9) x 10^5	(8.6±0.4) x 10^3	(1.7±0.1) x 10^4
5′-CCTTTTTCC-3′	(5.4±0.4) x 10^4	(4.2±0.5) x 10^4	(3.8±0.7) x 10^4	(5.6±0.7) x 10^4
5′-CCAATTTCC-3′	(3.0±0.2) x 10^5	(3.5±0.3) x 10^4	(3.3±0.2) x 10^4	(1.0±0.1) x 10^4

Binding constants were measured at 20°C in 1mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl.

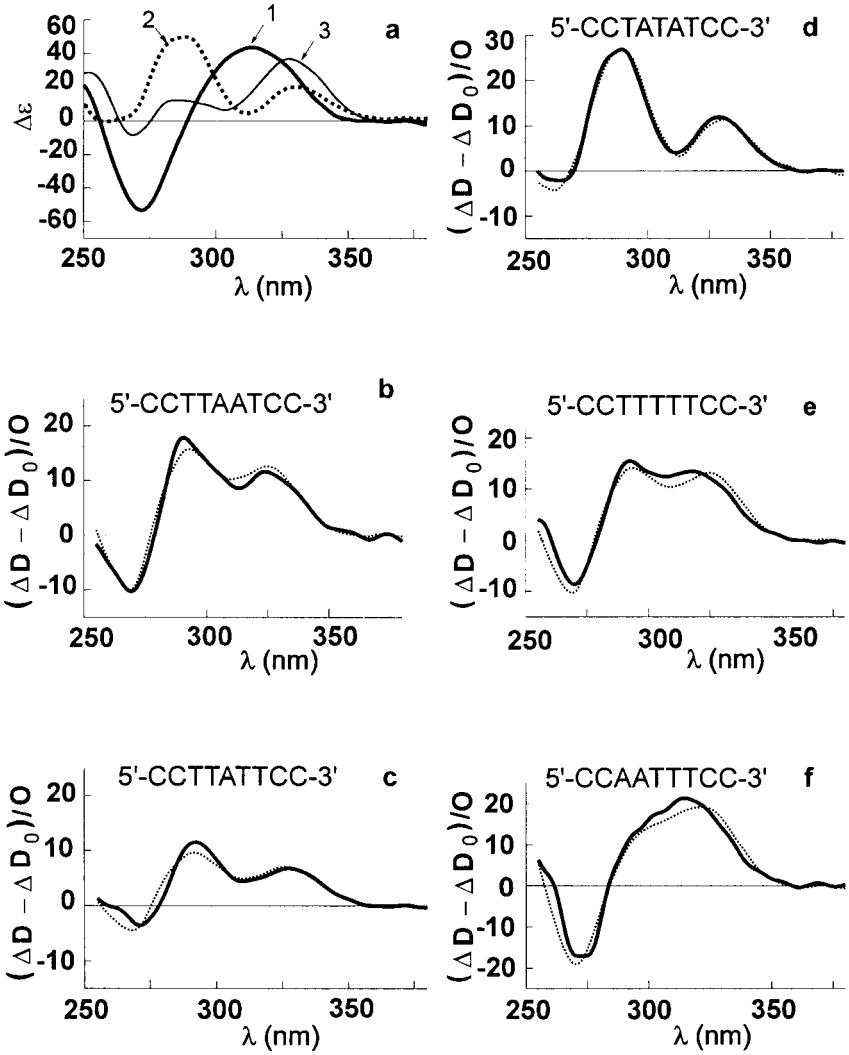

Figure 6: Decomposition of difference CD patterns for complexes of (←**Nt-(CH$_2$)$_5$-Nt**→) with DNA oligomers **II** (21.6 μM, panel *b*), **III** (20.4 μM, panel *c*), **I** (21.6 μM, panel *d*), **IV** (25.8 μM, panel *e*) and **V** (17.2 μM, panel *f*) into components which correspond to monodentate bis-netropsin binding and binding in the hairpin and self-associated dimer forms. Panel *a*, difference CD patterns characteristic of monodentate binding of ←**Nt-(CH$_2$)$_5$-Nt**→ (1) and binding in the hairpin (2) and dimer (3) forms. The C/O values were 2.10, 3.64, 2.78, 2.13 and 5.07 for complexes of ←**Nt-(CH$_2$)$_5$-Nt**→ with DNA oligomers **I**, **II**, **III**, **IV** and **V**, respectively. The experimental and calculated CD difference patterns are shown by solid and dotted lines, respectively. The best fit between the experimental and calculated CD patterns takes place when the occupancy of DNA oligomer by ←**Nt-(CH$_2$)$_5$-Nt**→ for monodentate binding mode, hairpin and dimer forms were as follows: panel *b*, R_1= 0.218 ± 0.004, R_2=0.300 ± 0.005, R_3=0; Panel *c*, R_1=0.098 ± 0.005, R_2= 0.188 ± 0.006, R_3= 0.030 ± 0.020; panel *d*, R_1=0.036 ± 0.005, R_2= 0.488 ± 0.005, R_3= 0; panel *e*, R_1=0.215 ± 0.007, R_2=0.253 ± 0.009, R_3= 0.085 ± 0.003 ; panel *f*, R_1= 0.356 ± 0.010; R_2=0.110 ± 0.014, R_3=0.258 ± 0.041.

The affinity constant for monodentate binding of ←**Nt-(CH$_2$)$_5$-Nt**→ to the 9-mer duplex with the sequence 5′- CCTATATCC - 3′ has been estimated to be 4 10^3 M^{-1} (Table I), and the ratio of the association constants corresponding to the hairpin and monodentate binding modes (K_2/K_1) is equal to 33 ±7 . This quantity appears to assume even a larger value for the system containing ←**Nt-Pt(NH$_3$)- Nt**→ and 9-mer duplex with the core sequence TATAT. In this case, deconvolution analysis yielded no indication for the occurrence of monodentate binding at the highest DNA oligomer concentration used (65 μM).

Looking at the results obtained from binding of ←**Nt-Pt(NH$_3$)- Nt**→ to DNA oligomers **II**, **III**, **IV** and **V**, one can conclude that ←**Nt-Pt(NH$_3$)- Nt**→ in the hairpin form binds strongly to duplexes **I** and **II** with the central sequences TATAT and TTAAT, respectively, and exhibits approximately 20 times lower affinity to the duplex **V,** containing the core sequence AATTT (Table I). The affinity order for binding of ←**Nt-Pt(NH$_3$)- Nt**→ in the hairpin form is 5′-CCTATATCC-3′ = 5′-CCTTAATCC-3′ >5′-CCTTATTCC-3′ >5′-CCTTTTTCC-3′ >5′-CCAATTTCC -3′. ←**Nt-(CH$_2$)$_5$-Nt**→ also exhibits a preference for binding in the hairpin form to duplex **I** and interacts less strongly with duplexes **IV** and **V**. Partially bonded monodentate binding of ←**Nt-(CH$_2$)$_5$-Nt**→ displays an opposite order of sequence preferences (Table I).

Discussion

Distinctly different CD spectral profiles observed for complexes of ←**Nt-Pt(NH$_3$)-Nt**→ and -**Nt**→**(Gly)$_3$-Nt**→ with 9-mer duplex 5′-CCTATATCC-3′ can be

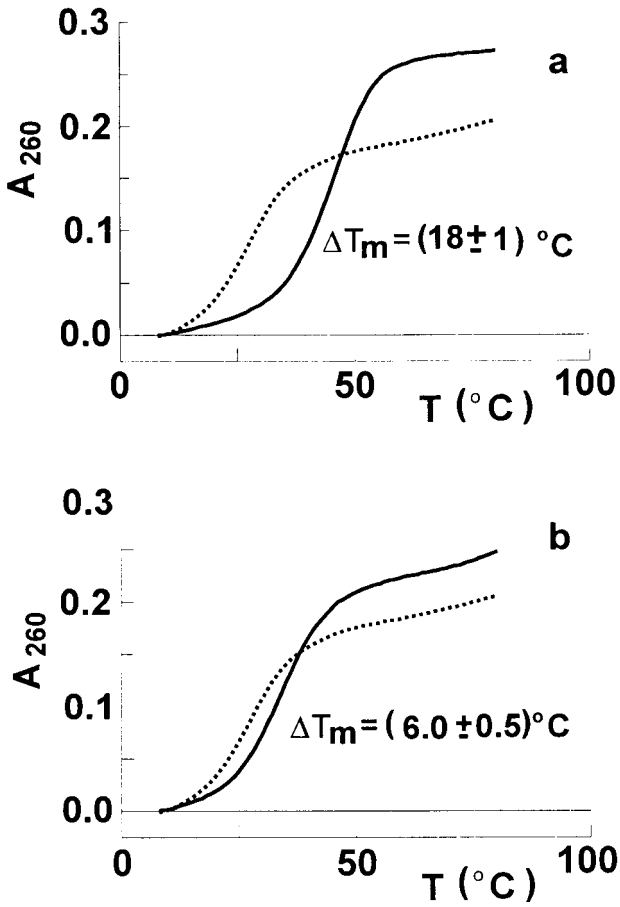

Figure 7: UV melting curves at 260 nm for DNA oligomer **I** (58 μM) and its complexes with ←**Nt-Pt(NH$_3$)-Nt**→ (panel *a*) and ←**Nt-(CH$_2$)$_5$-Nt**→ (panel *b*) at C/O values of 1.1 and 1.3 , respectively. Melting curves for the naked DNA oligomer and its complexes with synthetic ligands are shown by dotted and solid lines, respectively.

explained by differences in stacking interactions and local environment of two netropsin-like fragments combined in the parallel and antiparallel side-by-side manners in the minor DNA groove. Davydov splitting of the electron transitions corresponding to the absorbance bands of a netropsin analogue is expected for side-by-side arrangement of two netropsin-like fragments in the minor DNA groove, with the appearance of exciton type CD couplets in the difference CD spectral profiles. When a bis-netropsin binds to DNA in the extended conformation, stacking interaction and van-der-Waals contacts between two netropsin-like fragments are absent, and the CD spectra exhibit a single positive CD band with a peak at 314 nm. The CD spectra for complexes of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ with the 9-mer duplex exhibit two positive CD bands at 290 nm and 330 nm, with a negative CD amplitude at 310 nm. We suggest that this type of CD pattern reflects binding of ←**Nt-Pt(NH$_3$)$_2$-Nt**→ in the parallel-stranded hairpin form. Covalent head-to-tail linkage of two netropsin-like fragments is reflected in the appearance of distinctly different CD spectral profiles, as observed for complexes of **-Nt→(Gly)$_3$-Nt**→ with 9-mer and 10-mer duplexes with sequences 5′-CCTATATCC-3′ and 5′-CGTATATACG-3′, respectively.

Figure 8 shows the proposed molecular models for binding of an analogue of oligomethylene-bridged bis-netropsin (←**Nt-(CH$_2$)$_5$-Nt**→**)** in the extended conformation and parallel-stranded hairpin form to DNA dodecamer with the sequence 5′-CCTATATATACC-3′. The analogue is composed of N-methylpyrrole amino acid residues (Py) with the sequence NH_2-Py-Py-CO-$(CH_2)_5$-CO-Py-Py-NH_2 and has no charged dimethyl amino groups. Molecular modeling was carried out using modified Amber 4.1 force-field (39). For the electrostatic component of the empirical energy function the effect of solvent was approximated by a distance-dependent dielectric function and by reducing the net charge on the phosphate group to -0.46 e . It was assumed that bis-netropsin in the hairpin form occupies a central

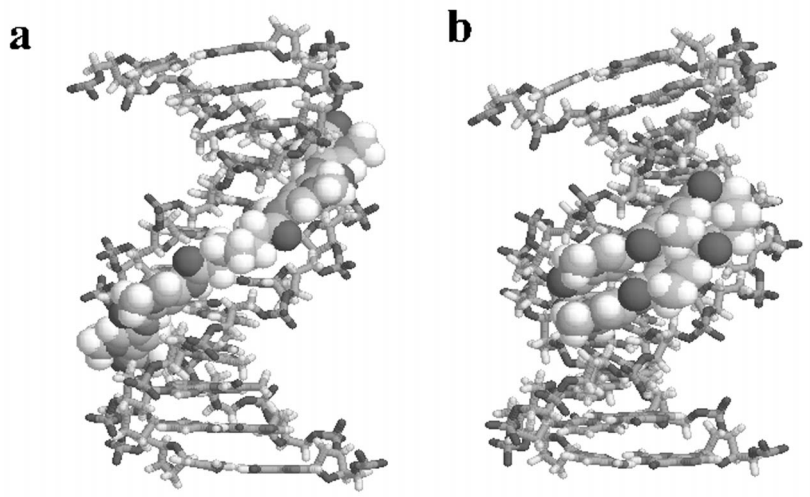

Figure 8: The proposed models for binding of analogue of ←**Nt-(CH$_2$)$_5$-Nt**→ in the extended conformation (*a*) and parallel-stranded hairpin form (*b*) to DNA.

position in the AT-track. In both structures the ligand sits deeply in the minor groove. DNA-ligand stabilization energy for interaction in the hairpin form is found to be approximately 1.2 times lower than that calculated for binding of the synthetic ligand in the extended conformation. In order to accommodate bis-netropsin in the parallel-stranded hairpin form the width of the minor DNA groove should be increased by approximately 2 A. In the complex, each of the two netropsin-like fragments of the hairpin polyamide is hydrogen bonded to adenine N3 and thymine O2 atoms lying on its own side of the groove.

The CD spectra of complexes of ←**Nt-(CH$_2$)$_5$-Nt**→ with the same 9-mer duplex were found to depend on concentration of the DNA oligomer in the titration assay. At DNA oligomer concentration as low as 1 10^{-5} M, the difference CD spectral profiles calculated per mole of bound bis-netropsin were found to be identical for binding of ←**Nt-Pt(NH$_3$)- Nt**→ and ←**Nt-(CH$_2$)$_5$-Nt**→ (data are not shown). They both exhibit two positive CD bands at 290 nm and 330 nm, with a negative CD contribution at 310 nm. At higher concentrations of the DNA oligomer the CD spectra exhibit the increased intensity at 290 nm, with a positive CD amplitude at 310 nm. One possible explanation for this behavior is that ←**Nt-(CH$_2$)$_5$-Nt**→ may have a higher flexibility as compared with ←**Nt-Pt(NH$_3$)- Nt** → and that several hairpin-like conformations of bound ligand may coexist at equilibrium . At low DNA oligomer concentrations, only a hairpin form with the highest affinity for DNA provides a substantial contribution into the induced CD signal.

The CD spectral profiles obtained for complexes of ←**Nt-(CH$_2$)$_5$-Nt**→ with DNA oligomers **II**, **III**, **IV** and **V** can be well represented as a superposition of three reference CD patterns corresponding to the monodentate bis-netropsin binding and binding in the parallel-stranded hairpin and antiparallel dimer forms. We have found that monodentate binding mode is favored relative to the hairpin form on binding of ←**Nt-(CH$_2$)$_5$-Nt**→ and ←**Nt-Pt(NH$_3$)- Nt**→ to the DNA oligomer **V**. Examination of CD spectral profiles obtained for complexes of ←**Nt-(CH$_2$)$_5$-Nt**→ with duplex **V** at C/O = 3.0 reveals that fractions of hairpin, antiparallel dimer and monodentate binding modes are 16, 39 and 53 %, respectively (see legend to Figure 6). Similar analysis for binding of ←**Nt-(CH$_2$)$_5$-Nt**→ to duplex **IV** at C/O = 2 shows that fractions of bound ligand corresponding to hairpin, dimer and monodentate binding modes are equal to 46, 15 and 39 %, respectively. This reflect the fact that hairpin form is favored relative to the 2:1 complex in a system containing ←**Nt-(CH$_2$)$_5$-Nt**→ and DNA oligomer **IV.**

Interesting the side-by-side dimer species were not detected in complexes between ←**Nt-(CH$_2$)$_5$-Nt**→ and duplex **II** which contains a TpA step and might have a widened minor groove (38). From NMR and crystallographic studies on binding of distamycin A to DNA oligomers with defined nucleotide sequences a conclusion

can be drawn that binding modes of distamycin A are strongly dependent on the base sequence [2,40,41]. For the sequence TATAT the 2:1 binding mode of distamycin is favored over the 1:1 complex. The sequence AAAAAA exhibits the opposite behavior - the 2:1 binding mode is disfavored relative to the 1:1 complex. The sequence AAATT exhibits an intermediate behavior between the two extreme cases mentioned above (41). Our present studies show that parallel-stranded hairpin form is favored relative to the 2:1 complex and monodentate binding mode in systems containing ←**Nt-$(CH_2)_5$-Nt**→ and DNA oligomers with central sequences TATAT and TTAAT and TTATT.

Self-associated dimer species were not observed in complexes of ←**Nt-Pt(NH_3)-Nt**→ with duplexes **I**, **II**, **III**, **IV** and **V**, even at high C/O values. One possible explanation for this is that electrostatic interaction between a positively charged dimethylamino group of one bis-netropsin molecule and positively charged cis-diammino platinum (II) group of the other molecule could destabilizes side-by-side interactions of two ligand molecules in the minor groove. The content of hairpin form is found to be correlated with the ratio of the binding constants K_2/K_1.

Quantitative DNAse I footprinting revealed that tetramers AATT and AAAA are better binding sites for netropsin, than TTAA and TATA (42). The presence of a TpA (but not ApT) step within an AT tract reduces binding affinity for netropsin . Sequence preferences for monodentate binding of ←**Nt-$(CH_2)_5$-Nt**→ were found to be similar to those shown by netropsin itself. The observed preferences of ←**Nt-Pt(NH_3)-Nt**→ and ←**Nt-$(CH_2)_5$-Nt**→ for binding in the hairpin form to the 9-mer duplexes with the core sequences TATAT, TTATT and TTAAT and their lower affinities to DNA oligomers containing sequences TTTTT and AATTT can be explained by particular conformation features of these DNA oligomers which contain TpA steps and might have a widened minor groove or increased flexibility. The wider minor groove is observed in the center of the dodecamer duplex d(CGCGTTAACGCG)$_2$ as compared with the width of the minor groove in the oligomer d(CGCGAATTCGCG)$_2$ (38). As any pyrimidine-purine step, the TpA step presents little overlap between adjacent base pairs. This allows bending via rolling in the major groove with concomitant widening of the minor groove. The TpA steps were identified as sites of substantial DNA bending in a number of protein-DNA complexes (43,44). Evidently, DNA oligomers with the narrow minor groove at the central AT tract serve as the best target sites for binding of netropsin and monodentate binding of ←**Nt-$(CH_2)_5$-Nt**→. However, an increased width of the minor groove is needed for binding in the groove of ←**Nt-Pt(NH_3)- Nt** → and ←**Nt-$(CH_2)_5$-Nt**→ in the parallel-stranded hairpin form.

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