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Hairpin Polyamides That use Parallel and Antiparallel Side-by-Side Peptide Motifs in Binding to DNA

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Abstract

Pt-bis-netropsin is a synthetic sequence-specific DNA-binding ligand comprizing two netropsin-like fragments which are linked in a tail-to-tail manner via a *cis*-diammineplatinum (II) residue. The CD studies and thermodynamic characterization of the DNA-binding properties exhibited by this compound reveal that it forms two types of complexes with poly[d(AT)]•poly[d(AT)] and DNA oligomers containing nucleotide sequences 5'-CC(TA)$_n$ CC-3', with n = 4, 5 and 6. The first type corresponds to the binding of Pt-bis-netropsin in the extended conformation and is characterized by the saturating ratio of one bound Pt-bis-netropsin molecule per 9 AT-base pairs. The second type of the complex corresponds to the binding of Pt-bis-netropsin to DNA in the folded hairpin form. The binding approaches saturation level when one Pt-bis-netropsin molecule is bound per four or five AT-base pairs. The hairpin form of Pt-bis-netropsin complex is built on the basis of parallel side-by-side peptide motif which is inserted in the minor DNA groove. The CD spectral profiles reflecting the binding of Pt-bis-netropsin in the hairpin form are different from those observed for binding of another bis-netropsin with the sequence Lys-Gly-Py-Py-Gly-Gly-Gly-Py-Py-Dp, where Py is a N-propylpyrrole amino acid residue and Dp is a dimethylaminopropylamino residue. The hairpin form of this bis-netropsin is formed on the basis of antiparallel side-by-side peptide motif. The CD spectra obtained for complexes of this polyamide in the hairpin form with poly[d(AT)]•poly[d(AT)] exhibit positive CD band with a peak at 325 nm, whereas the CD spectral profiles for the second complex of Pt-bis-Nt with poly[d(AT)]•poly[d(AT)] and short DNA oligomers have two intense positive CD bands near 290 nm and 328 nm. This reflects the fact that two bis-netropsins use different structural motifs on binding to DNA in the hairpin form.

Introduction

Netropsin (Figure 1) and its analog distamycin A are natural antibiotics that contain two or three N-methylpyrrolcarboxamide groups and bind in the minor groove of double-helical DNA at sites of 4 or 5 successive AT- base pairs (for a review see ref. 1). X-ray (2-4) and NMR studies (5) of netropsin-DNA and distamycin-DNA complexes show that pyrrole rings of the drug molecules are inserted into the minor groove and the carboxamide NH groups form hydrogen bonds with the N3 of adenine and the O2 of thymine, in agreement with the molecular models suggested for these complexes earlier (6,7, see also reference 1). Previous crystal structures of netropsin-DNA and distamycin-DNA complexes have a stoichiometry 1:1 (2,3). Recent studies revealed that two distamycin molecules can bind to the same region of poly[d(AT)]•poly[d(AT)] in an antiparallel side-by-side manner (8-11). This model was first proposed by Burckhardt et al. from studies on binding of distamycin A analogs to poly[d(AT)]•poly[d(AT)] (8), but structural information was lacking, until Wemmer and coworkers carried out NMR studies of the distamycin complex with an undecamer duplex (9,10). Recently the crystal structure of a 2:1 complex between distamycin A and an inosine-containing alternating octamer duplex, d(ICICICIC)$_2$, has been determined (11). In this complex, the distamycin molecules are inserted in the minor groove in a side-by-side maner, with the pyr-

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role rings of one antibiotic molecule stacking on the peptide groups of the other. Each bound distamycin molecule is hydrogen bonded to acceptor sites represented by cytosine O2 or inosine N3 atoms lying along its own side of the groove. The side-by-side antiparallel peptide motif was used by Dervan and Lown and their coworkers for the design of a new generation of sequence-specific DNA-binding ligands containing pyridine-2-carboxamide or 1-methylimidazole-2-carboxamide instead of a N-methylpyrrole-carboxamide group in a netropsin analog (12-16). It was shown that two ligands, distamycin A and 1-methylimidazole-2-carboxamidonetropsin, can bind simultaneously in the minor groove to the five base pair sequence 5'-TGTTA-3' as a side-by-side heterodimer (15). Geierstanger et al. have reported that a four-ring analog, with the sequence imidazole-pyrrole-imidazole-pyrrole, binds preferentially to DNA regions with a sequence 5'-(A,T)GCGC(A,T)-3' as a dimer (17). Earlier, synthesis of DNA-binding ligands composed of two netropsin analogs bridged by flexible linkers in tail-to-head, head-to-head and tail-to-tail manners have been reported (18). These compounds exhibit a high binding specificity and inhibit selectively initiation of transcription directed by certain procaryotic promoters (18). Recently it was shown that a synthetic ligand composed of two netropsin analogs linked via a γ-aminobutyric acid residue can bind in the DNA minor groove in the extended and folded hairpin conformations, using different DNA-binding motifs (19-21). The hairpin form of this polyamide is built on the basis of antiparallel side-by-side peptide motif.

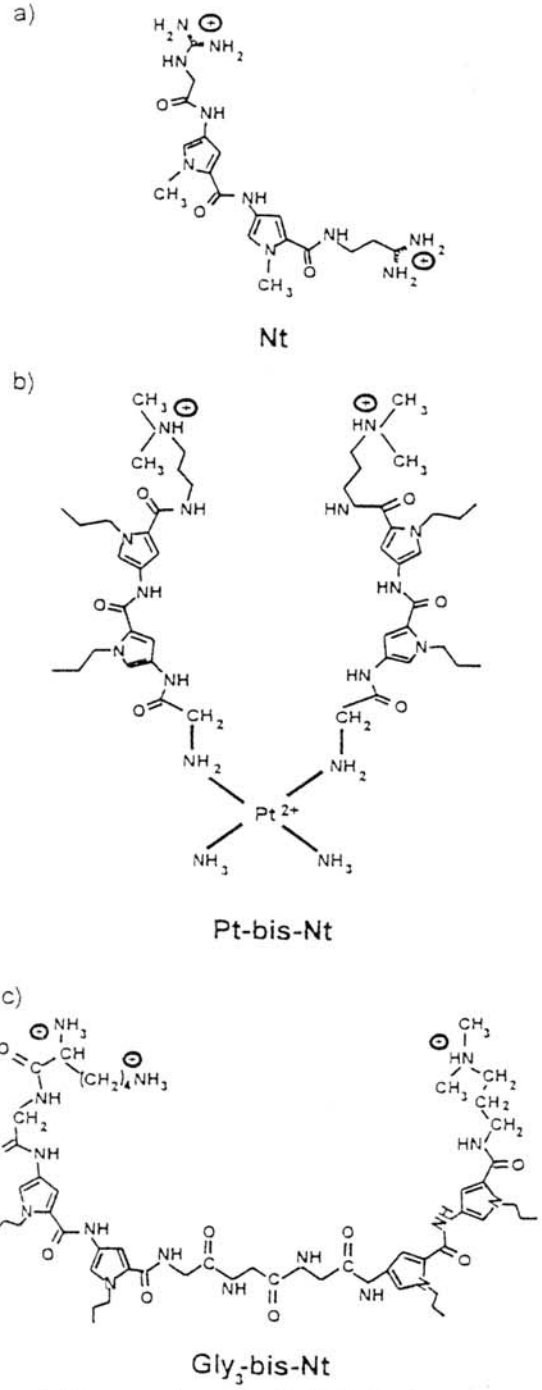


Figure 1: Structures of netropsin (a), **Pt-bis Nt** (b) and Gly_3-bis-Nt (c).
Each bis-netropsin contains two netropsin-like fragments bridged by short linkers in the tail to tail (b) and tail to head (c) manners.

In the present work, we report on the DNA-binding properties of two sequence-specific DNA-binding ligands composed of two netropsin-like fragments bridged via short linkers in a tail-to-tail and tail-to-head orientation (Figure 1). One of them with the sequence Dp-Py-Py-Gly-*cis*[$Pt(NH_3)_2$]$^{2+}$-Gly-Py-Py-Dp will be referred to as the Pt-bis-netropsin (**Pt-bis-Nt**). Here Py is a N-propylpyrrole amino acid residue. *Cis*[$Pt(NH_3)_2$]$^{2+}$ is a *cis*-diammineplatinum (II) residue. Dp is a di-methylaminopropylamino group. From previous studies it is known that **Pt-bis-Nt** binds at selective sites on DNA. After X-ray irradiation of **Pt-bis-Nt**-DNA complexes a platinum-mediated DNA cleavage of DNA is observed at preferred binding sites for **Pt-bis-Nt** on DNA (22). To obtain further information on binding of **Pt-bis-Nt** to DNA we were interested to compare the DNA binding properties of **Pt-bis-Nt** with those of a new bis-netropsin analog with the sequence Lys-Gly-Py-Py-Gly-Gly-Gly-Py-Py-Dp. This polyamide will be referred to as the Gly_3-bis-netropsin (**Gly_3-bis-Nt**). We found that each of the two bis-netropsins forms only one type of complex with poly(dA)•poly(dT). However, **Pt-bis-Nt** and **Gly_3-bis-Nt** form two types of complexes with poly[d(AT)]•poly[d(AT)] which correspond to the binding of each bis-netropsin in the monomer form in the extended and folded hairpin conformation. The CD data and molecular model building studies suggest that two netropsin-like fragments of the **Pt-bis-Nt** molecule can be sandwiched in the minor groove of poly[d(AT)]•poly[d(AT)] with pyrrole rings of one fragment stacking on pyrrole rings of the other. The difference in stacking and environment of two netropsin-like fragments combined in side-by-side parallel and antiparallel motifs in the DNA minor groove might be responsible for the observed difference in CD patterns for complexes of these two bis-netropsins with poly[d(AT)]•poly[d(AT)].

The binding of two bis-netropsins to DNA oligomers containing sequences 5'- $CC(TA)_n$- CC-3', with n = 4, 5 and 6, respectively, was also studied. Our experiments show that **Pt-bis-Nt** and **Gly_3-bis-Nt** bind to these oligonucleotide duplexes in the extended and hairpin forms, in close similarity with the results obtained for their binding to poly[d(AT)]•poly[d(AT)]. However, in a

system containing short DNA duplex, an equilibrium between the extended and hairpin form is shifted toward the formation of hairpin form.

Materials and Methods

Pt-bis-Nt (see Figure 1) was synthesized and purified as described previously (23). Synthesis of **Gly_3-bis-Nt** will be published elsewhere. The concentration of **Pt-bis-Nt** and **Gly_3-bis-Nt** were determined spectrophotometrically using the molar extinction coefficient at 297 nm of 42,000 M^{-1} cm^{-1}. Poly(dA)•poly(dT) (ε_{260} = 12,000 M^{-1} cm^{-1}) and poly[d(AT)]•poly[d(AT)] (ε_{260} = 13, 600 M^{-1} cm^{-1}) were obtained from P.L. Biochemicals and used without further purification. The molar absorbances for all polynucleotides are expressed per mole of base pair. Polynucleotides were dissolved in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl and 1 mM EDTA and then dialyzed over 24 h against the same buffer in the absence of EDTA. Spectrophotometric measurements were carried out on a Cary1E instrument. The CD spectra were recorded on a Jasco Model 720 instrument using 1.0, 0.2 and 0.1 cm pathlength cells. Oligonucleotides were synthesized on the basis of phosphoramidite chemistry by using of an automated synthesizer from Applied Biosystems. The following oligonucleotides were used:

5'-CCTATATATACC-3'	(I)	5'-GGTATATATAGG-3'	(II)
5'-CCTATATATATACC-3'	(III)	5'-GGTATATATATAGG-3'	(IV)
5'-CCTATATATATATACC-3'	(V)	5'-GGTATATATATATAGG-3'	(VI)

Molar concentrations of oligonucleotides I to VI were estimated by measuring optical densities of oligonucleotide solutions at 90° C in 260 nm, using the molar extinction coefficients at 260 nm of 128,000, 145,600, 152,600, 170,200, 177,200 and 194,800 $M^{-1}cm^{-1}$ for oligonucleotides I, II , III, IV ,V and VI, respectively. Annealing of complementary strands to form duplexes containing oligonucleotides I+II , III+IV and V+VI was accomplished by heating the solution containing complementary oligonucleotides in equimolar quantities and then cooling over-night to the room temperature. The melting curves obtained for duplexes exhibit only one transition of the double-helical oligomer. The duplex and single-stranded hairpin can be distinguished with an aid of ethidium bromide fluorescence assay and measure-ments of the degree of fluorescence polarization for complexes of ethidium bromide with DNA oligomers at low ethidium bromide/DNA oligomer molar ratios (< 0.1). The magnitude of the rotational fluorescence depolarization of bound ethidium bromide is known to depend on the molecular mass and shape of the oligonucleotide species present in solution. The results of measurements of the degree of polarization of ethidium bromide fluorescence and shapes of the experimental melting curves for DNA oligomers are consistent with the presence of duplex form only.

Results and Discussion

*Binding of **Pt-bis-Nt** and **Gly_3-bis-Nt** to poly(dA)• poly(dT) and poly[d(AT)]• poly[d(AT)]*

The binding of **Pt-bis-Nt** to synthetic poly- and oligodeoxyribonucleotides can be monitored by CD spectroscopy in the wavelength region of 300-350 nm. Figure 2 shows the CD spectra of complexes formed by **Pt-bis-Nt** with poly(dA)• poly(dT). The maximum in the CD band occurs at 316 nm with a molar CD absorbance ($\Delta\varepsilon_{316}$) of about 10.5 per mole of base pairs. The CD spectra recorded at different molar ratios of **Pt-bis-Nt** to DNA base pairs (C/BP) are very similar. Here C is the concentration of **Pt-bis-Nt**. BP is the concentration of nucleic acid expressed in moles of base pairs. The CD spectra exhibit isodichroic points at 204, 254 and 285 nm. This suggests that only one type of complex is formed between **Pt-bis-Nt** and poly(dA)•poly(dT) under the experimental conditions used. Since the free **Pt-bis-Nt** is found to be optically inactive, the CD amplitudes at 310 and 340 nm

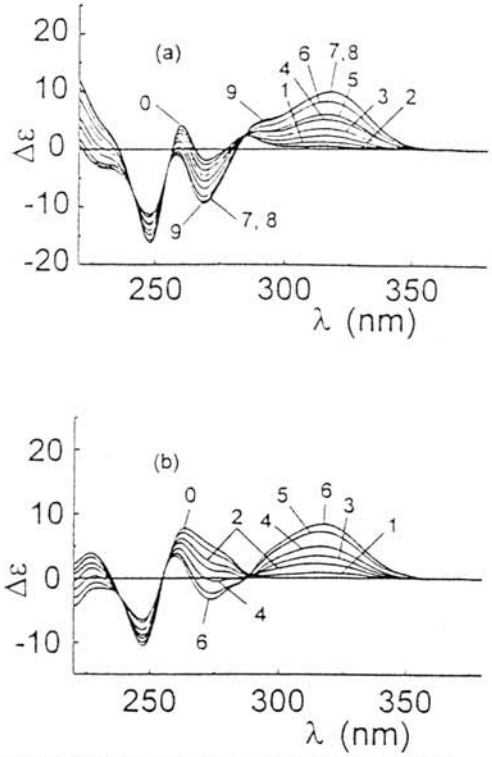


Figure 2: CD spectra of complexes between Pt-bis-Nt and (a) poly(dA)•poly(dT) (51 μM base pairs) and (b) poly[d(AT)]•poly[d(AT)] (45 μM base pairs) at different molar ratios of **Pt-bis-Nt** to DNA base pairs (C/BP). The C/BP values were as follows: panel a, 0 (0), 1 (0.01), 2 (0.02), 3 (0.03), 4 (0.04), 5 (0.05), 6 (0.08), 7 (0.11), 8 (0.15), 9 (0.27); panel b, 0 (0), 1 (0.01), 2 (0.02), 3 (0.04), 4 (0.06), 5 (0.08), 6 (0.1). The CD spectra were recorded in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl at 20° C.

are proportional to the amount of bis-netropsin bound to DNA.

Figure 4 shows typical CD titration curves obtained from binding of **Pt-bis-Nt** to poly(dA)•poly(dT). The binding approaches saturaton when one Pt-bis-netropsin molecule is bound per 11 base pairs of poly(dA)•poly(dT). Since netropsin occupies 5 base pairs upon binding to poly(dA)•poly(dT) [1,6] we interprete these observa- tions as indicating that each bound bis-netropsin molecule extends over one turn of the DNA helix in the minor groove and that two netropsin-like fragments of the bis-netropsin molecule are implicated in specific interaction with AT-base pairs of poly(dA)•poly(dT). The molar dichroism of bis-netropsin complex at 310 nm and 340 nm can be determined from the initial slopes of the titration curves obtained in the presence of large molar excess of DNA over **Pt-bis-Nt**, when practically all bis-netropsin molecules are bound to a nucleic acid. We found that molar dichroism value of **Pt-bis-Nt** complex with poly(dA)•poly(dT) at 310 nm expressed per mole of binding sites is equal to 95±5. Each binding site is spanning over 10 to 11 base pairs. Similar results were obtained for binding of **Gly_3-bis-Nt** to poly(dA)•poly(dT) (data not shown).

In contrast to the binding to poly(dA)•poly(dT), **Gly_3-bis-Nt** and **Pt-bis-Nt** form two types of complexes with poly[d(AT)]•poly[d(AT)]. The first type is characterized by CD spectral profile similar to that observed for binding of each bis-netropsin to poly(dA)•poly(dT) (Figure 2). This complex is formed at relatively low **Pt-bis-Nt**/DNA base pair ratios (C/BP< 0.10), when CD spectra exhibit an isodichroic point at 290 nm (Figure 2, b). Increasing the C/BP value from 0.1 to 0.2 leads to a considerable change in the CD spectral profile in the region of 300-360 nm where the contribution of DNA is negligible. The CD amplitude at 310 nm decreases when C/BP value increases from 0.1 to 0.2 (Figure 4), the isodichroic point at 290 nm dissappears, the maximum of the CD band is shifted to a longer wavelength region by approximately 10 nm, and a new isodichroic point at 320.5 nm appears. These spectral changes reflect formation of a second type of the complex between **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)].

Similar spectral changes are observed for complexes of **Gly_3-bis-Nt** with poly[d(AT)]•poly[d(AT)] at C/BP values approaching the saturation level of binding (Figure 3, a). At low C/BP values (C/BP<0.1) the CD spectra exhibit a positive band with a peak at 316 nm. Increasing the C/BP value from 0.15 to 0.50 leads to

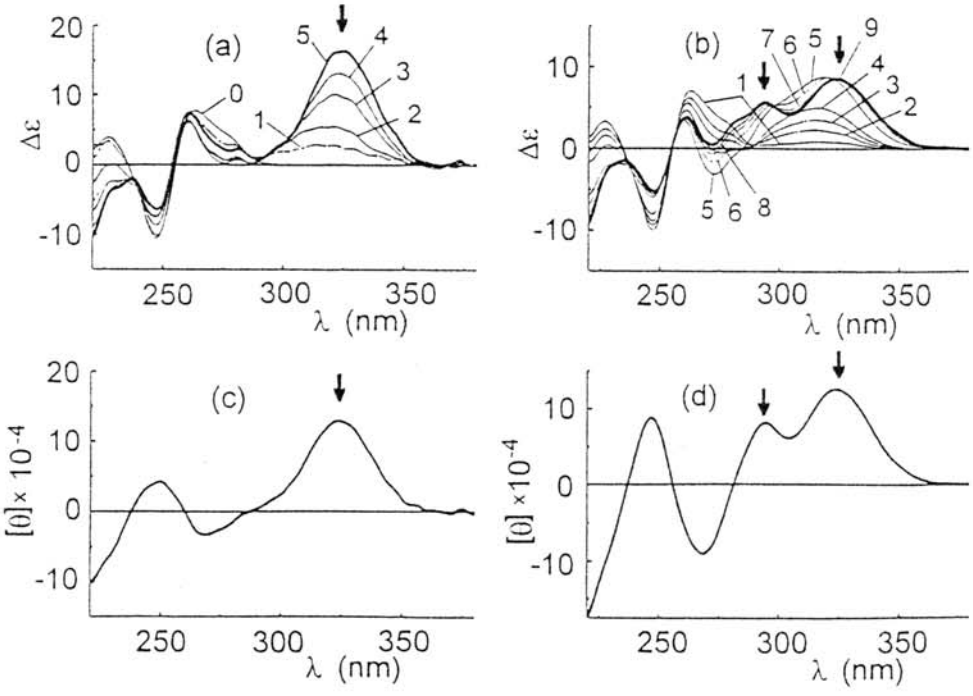


Figure 3: CD spectra obtained for mixture of poly[d(AT)]•poly[d(AT)] (62.5 μM base pairs) with **Gly_3-bis-Nt** (a) and for mixture of poly[d(AT)]•poly[d(AT)] (45 μM base pairs) with **Pt-bis-Nt** (b). The C/BP values were as follows: (panel a), 0 (0), 1 (0.03), 2 (0.08), 3 (0.16), 4 (0.26), 5 (0.61); (panel b), 0 (0), 1 (0.01), 2 (0.02), 3 (0.04), 4 (0.06), 5 (0.13), 6 (0.16), 7 (0.19), 8 (0.25), 9 (0.40). Difference CD spectra calculated by subtraction of the CD spectra of the nucleic acid alone from the spectra of complexes of **Gly_3-bis-Nt** (c) and **Pt-bis-Nt** (d) with poly[d(AT)]•poly[d(AT)] at C/BP value equal to 0.6 and 0.4, respectively. [θ] is the molar ellipticity in deg cm² dmol⁻¹ (per decamole of bound ligand). Arrows indicate positions of characteristic CD maxima. Conditions are the same as in Figure 2.

considerable changes in the CD spectral profile in the region of 300-360 nm. At C/BP values > 0.15 a positive CD band with a peak at 325 nm is observed.

The difference CD spectra calculated by subtraction of the CD spectra of the nucleic acid component alone from the spectra of the bis-netropsin- DNA mixtures are shown in Figure 3. Concentrations of bound bis-netropsin were calculated from measurements of CD amplitude at 320.5 nm (isodichroic point) which is proportional to the amount of bis-netropsin bound to a nucleic acid. In the difference CD spectra shown in Figure 3 the CD amplitude is divided on the molar concentration of bound **Pt-bis-Nt** in the titration assay. One can see that difference spectra obtained for complexes of the second type between **Pt-bis-Nt** and **Gly_3-bis-Nt** and

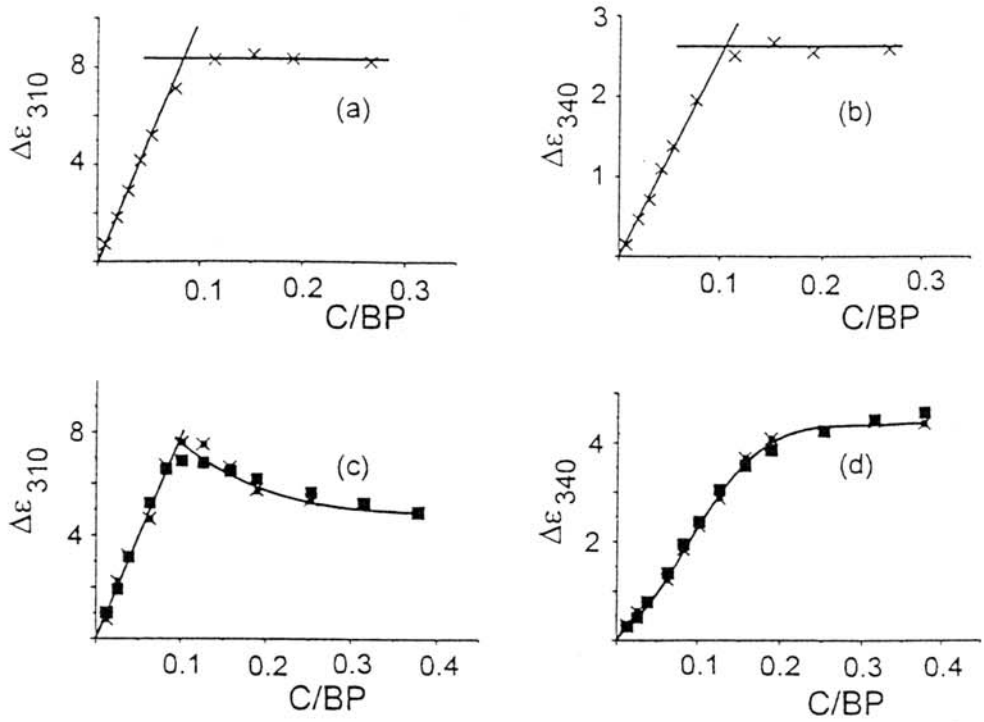


Figure 4: Titrations of (a,b) poly(dA)•poly(dT) (51 μM base pairs) and (c,d) poly[d(AT)]•poly[d(AT)] (45 μM base pairs) by **Pt-bis-Nt** at 310 and 340 nm. $\Delta\varepsilon_{310}$ and $\Delta\varepsilon_{340}$ are the CD amplitudes measured at 310 and 340 nm, respectively, and expressed per mole of base pairs and 1 cm pathlength cell.

×, experimental data points; ■, theoretical values of Δε calculated from Eqs. 1-4 at different values of C/BP using the best fit values for binding parameters: panel c, $K_1 = 31.6\times10^6\ M^{-1}$, $K_2 = 1.0\times10^6\ M^{-1}$; $L_1 = 9$ and $L_2 = 4$; panel d, $K_1 = 2.9\times10^7\ M^{-1}$, $K_2 = 3.5\times10^6\ M^{-1}$, $L_1 = 9$ and $L_2 = 4$. Conditions are the same as in Figure 2.

poly[d(AT)]•poly[d(AT)] are different. The CD spectra for **Pt-bis-Nt**-DNA complex exhibit two positive CD bands near 290 and 325 nm, whereas only one positive band near 325 nm is observed for complexes with **Gly_3-bis-Nt.**

Figure 4 shows titration curves obtained from binding of **Pt-bis-Nt** to poly(dA)•poly(dT) and poly[d(AT)]•poly[d(AT)]. The CD amplitudes at 310 and 340 nm were measured as functions of molar ratio of added bis-netropsin to DNA base pairs (C/BP). In the initial stage of titration corresponding to low values of C/BP (C/BP< 0.1) the CD amplitude at 310 nm is found to increase until C/BP value approaches the saturation level of binding for the first complex, beyond which the CD amplitude decreases, thereby reflecting the formation of a second complex between **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)] (Figure 4, c). Formation of a second complex can be easily detected by comparing the CD titration curves obtained at 310 and 340 nm. The contribution of a second complex to an overall CD amplitude at 340 nm is comparable to that of the first complex. As can be seen from Figure 4, the CD amplitude at 340 nm approaches a plateau level at C/BP = 0.20 which corresponds to 2:1 molar ratio of bound **Pt-bis-Nt** molecules per DNA region extending over 10 base pairs. This is in contrast to the behavior observed for binding of **Pt-bis-Nt** to poly(dA)•poly(dT). In this case the binding approaches saturation at C/BP value approximately equal to 0.1, irrespective of whether CD titrations were carried out at 310 or 340 nm.

Figure 5 shows titrations curves obtained for binding of **Gly_3-bis-Nt** to

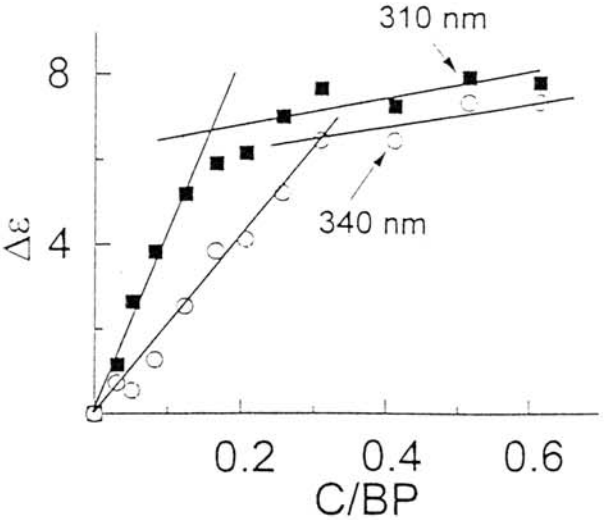


Figure 5: Titration of poly[d(AT)]•poly[d(AT)] (62.5 μM base pairs) by Gly_3-bis-Nt at 310 nm and 340 nm. Conditions are the same as in Figure 2.

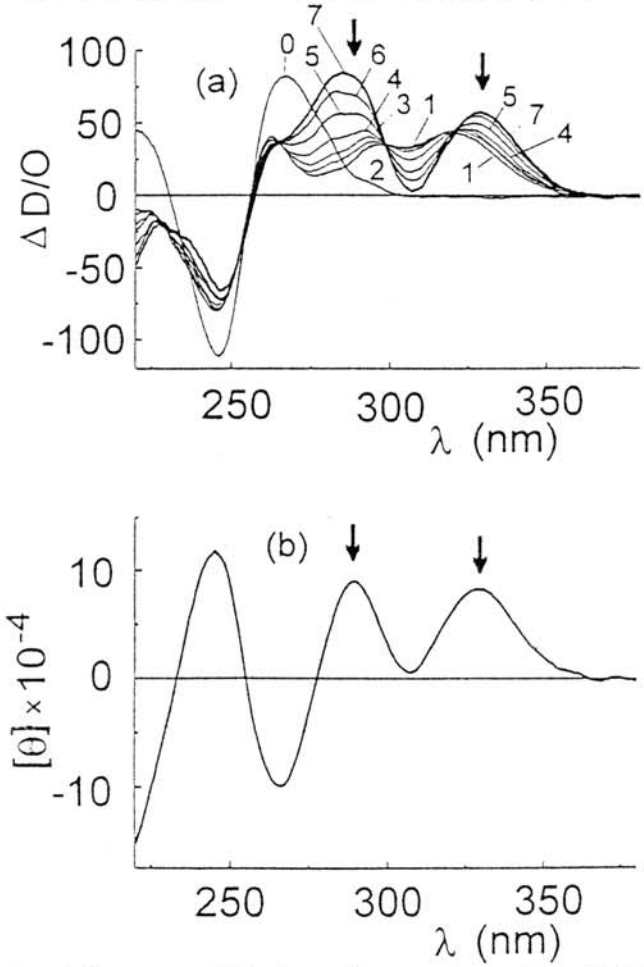


Figure 6: CD spectra recorded for mixtures of Pt-bis-Nt and 14-mer duplex (21 μM) at different molar ratios of Pt-bis-Nt to the oligonucleotide duplex (a). The C/O values are as follows: 0 (0), 1 (1.15), 2 (1.26), 3 (1.41), 4 (1.67), 5 (1.94), 6 (2.35), 7 (3.40). Difference CD spectra obtained at C/O = 3.4 (panel b). ΔD/O is the CD amplitude calculated per 1 cm pathlength cell and one mole of the DNA oligomer. [θ] is the molar ellipticity in deg cm² dmol⁻¹ (per decamole of bound ligand). Arrows indicate positions of characteristic CD maxima. Conditions are the same as in Figure 2.

poly[d(AT)]•poly[d(AT)]. As revealed from the position of the breakpoint in the titration curve measured at 310 nm, complex of the first type approaches saturation when C/BP ≈ 0.15. Formation of complex of the second type approaches saturation at approximately two times higher C/BP value.

Binding of ***Pt-bis-Nt*** *and* ***Gly_3-bis-Nt*** *to synthetic DNA oligomers.*

Similar results are obtained for binding of **Pt-bis-Nt** and **Gly_3-bis-Nt** to short DNA oligomers with sequences 5'-CCTATATATACC-3', 5'-CCTATATATATACC-3' and 5'-CCTATATATATATACC-3' (data not shown). Figure 6 shows CD spectra for complexes of **Pt-bis-Nt** with the 14-mer duplex 5'-CCTATATATATACC-3'. The spectra exhibit well-defined isodichroic points at 320.5 and 298 nm and show two intense positive CD bands near 288 nm and 328 nm which are characteristic of a second type of the complex between **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)]. The difference CD spectra calculated by subtraction of the CD spectrum of the oligonucleotide duplex alone from the spectra of the bis-netropsin- oligonucleotide complexes are shown in Figure 6. The difference spectra obtained for systems containing poly[d(AT)•poly[d(AT)] and DNA oligomers are similar, in spite of differences in the nucleotide sequence and composition. In the 250-350 nm spectral region the difference CD patterns exhibit two positive bands at 288 and 328 nm. These CD patterns are obtained at C/BP values approaching the saturation level of binding when the contribution of the complex of the first type was small.

We suggest that **Pt-bis-Nt** binds to poly[d(AT)]•poly[d(AT)] and the 14-mer duplex in an extended and hairpin-like form and interacts only in the extended form with poly(dA)•poly(dT). Interesting, that only CD patterns characteristic of complex of the second type with a peak at 328 nm are observed for complexes of **Pt-bis-Nt** with the DNA oligomer 5'-CCTATATATACC-3', whereas the CD spectra typical of complex of the first type with a peak at 314 nm were not observed (Figure 7), even at very low **Pt-bis-Nt**/ DNA oligomer ratios (C/O<0.03, where O is the DNA oligomer concentration, C is the concentration of **Pt-bis-Nt**). This means that in this system an equilibrium between the extended and hairpin-like form of the bis-netrop-sin complex is shifted toward the formation of the hairpin form.

Similar results are obtained for binding of **Gly_3-bis-Nt** to the same 12-mer duplex. In this case the CD spectra exhibit only one positive band near 325 nm which is characteristic of binding in the hairpin form (Figure 7). The CD spectra typical of complex of the first type with a peak at 316 nm are not observed, even at very low **Gly_3-bis-Nt**/DNA oligomer molar ratios.

Figure 8 shows typical CD titration curves obtained from binding of **Pt-bis-Nt** to the 14-mer duplex. As before, CD titrations were carried out at 310 and 340 nm. As follows from the shapes of the titration curves, formation of first type of the complex between **Pt-bis-Nt** and 14-mer duplex has a stoichiometry of 1:1, whereas the binding of **Pt-bis-Nt** in the hairpin form is characterized by a stoichiometry of 2:1. Complexes of **Pt-bis-Nt** and **Gly_3-bis-Nt** with the 12-mer duplex have also stoichiometries of 2:1(data not shown).

Thermodynamic characterization of complexes formed by **Pt-bis-Nt** *with poly[d(AT)]•poly[d(AT)] and synthetic DNA oligomers.*

Our next step was to determine thermodynamic parameters for bind-

ing of **Pt-bis-Nt** in the extended and hairpin conformation to poly[d(AT)]•poly[d(AT)] and short DNA duplexes with sequences 5'-CC(TA)$_n$CC-3' (n is equal to 4, 5 and 6). Let K_1 and K_2 be the equilibrium association constants of the bis-netropsin binding in the extended and hairpin forms, respectively, to an isolated site on the polynucleo-tide lattice. It is assumed that each bis-netropsin molecule occupies L_1 and L_2 consecutive base pairs when bound in the extended and hairpin form, respectively. It can be shown that noncooperative ligand binding to a homogeneous polynucleotide lattice can be described by Eqs. [1], [2] and [3] (24,25).

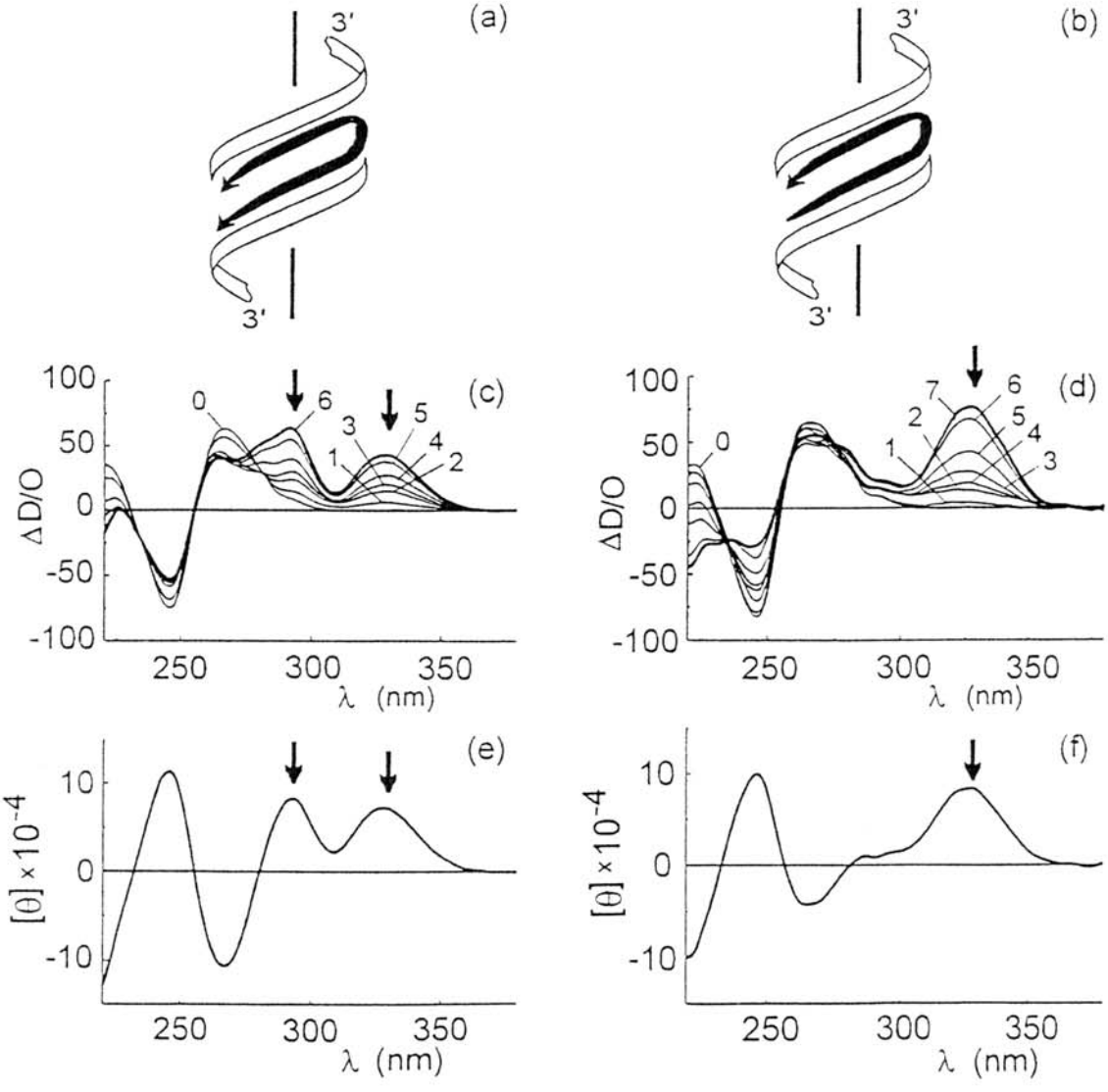
$$r_1 = K_1 m(1 - r_1 L_1 - r_2 L_2)^{L_1} / (1 - r_1 L_1 - r_2 L_2 + r_1 + r_2)^{L_1 - 1} \quad [1]$$

$$r_2 = K_2 m(1 - r_1 L_1 - r_2 L_2)^{L_2} / (1 - r_1 L_1 - r_2 L_2 + r_1 + r_2)^{L_2 - 1} \quad [2]$$

$$m = C - (r_1 + r_2)BP \quad [3]$$

where r_1 and r_2 are the molar ratios of bis-netropsin bound in the extended and hairpin form, respectively, to DNA base pairs. m is the concentration of the free bis-netropsin. C is the total concentration of ligand in solution. BP is the concentration of DNA expressed in moles of base pairs.

Figure 7: Comparison of CD spectral profiles observed for complexes of hairpin polyamides with 12-mer duplex. General plans of the complex structures formed by **Pt-bis-Nt** (a) and **Gly$_3$-bis-Nt** (b) with DNA in the hairpin forms. The hairpins are built on the basis of parallel and antiparallel side-by-side peptide motifs, respectively. The CD spectra obtained for mixtures of **Pt-bis-Nt** and 12-mer duplex (30 μM) (c) and for mixtures of **Gly$_3$-bis-Nt** and 12-mer duplex (32.6 μM) (d). The C/O values are as follows: panel c, 0 (0), 1 (0.08), 2 (0.33), 3 (0.49), 4 (0.82), 5 (1.65), 6 (2.14); panel d, 0 (0), 1 (0.15), 2 (0.31), 3 (0.53), 4 (0.88), 5 (1.32), 6 (2.41), 7 (2.87). The difference CD spectra calculated for mixture of **Pt-bis-Nt** and 12-mer duplex at C/O = 4.1 (e) and for mixture of **Gly$_3$-bis-Nt** and 12-mer duplex at C/O = 4.4 (f). Conditions are the same as in Figure 2.



In order to determine thermodynamic parameters from experimental titration curves measured at 310 and 340 nm, it is convenient to compare the experimental and theoretically calculated plots of $\Delta\varepsilon$ versus C/BP (Figure 4, c and d). Here $\Delta\varepsilon$ is the CD amplitude of the bis-netropsin-DNA complex measured at a given wavelength and calculated per 1 cm pathlength cell and one mole of DNA base pairs. If only two bound bis-netropsin species are present in solution, then

$$\Delta\varepsilon = r_1\Delta\varepsilon_1 + r_2\Delta\varepsilon_2 \qquad [4]$$

where De_1 and De_2 are the molar dichroism values for the first and second complex of **Pt-bis-Nt** with DNA, respectively. The molar dichroism value for the first complex can be determined from measurements of the CD amplitude in the presence of large molar excess of poly[d(AT)]•poly[d(AT)] over the ligand. From the initial slope of the plot similar to that shown in Figure 4 we found that the molar dichroism value for the first complex at 310 nm is equal to 85±5. At high levels of binding one may, in the first approximation, neglect contribution of the first complex to the CD signal and estimate the molar dichroism value for the second complex between **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)]. In order to accomplish this we also used data obtained for binding of **Pt-bis-Nt** to DNA oligomers with sequences 5'-CC$(TA)_n$CC-3', where n is 4, 5 and 6. In these systems, the equilibrium between the extended and hairpin form is shifted toward the formation of hairpin form. The molar dichroism value at 310 nm for the second complex between **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)] is found to be 4±1.

The theoretical plots of $\Delta\varepsilon$ versus C/BP were calculated from Eqs. [1], [2], [3] and [4] for different values of K_1 and K_2 and site sizes equal to 9 and 10 (extended form) and 4 or 5 (hairpin form), using experimentally determined values of $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ at 310 and 340 nm. Thermodynamic parameters were determined by a combination of an iterative and nonlinear least squares data-fitting procedure in which the minimum of the mean square deviation between the experimental and theoretical values of $\Delta\varepsilon$ at different C/BP values was taken as a criterion of the quality of the fit between the experimental and calculated curves. The data-fitting procedure used in the present work exhibits some resemblance with that outlined earlier for a different thermodynamic model (26). At the first round of calculations, we suggested that there is only one bound bis-netropsin species and calculated concentrations of free and bound bis-netropsin from measurements of the CD amplitude at 320.5 nm where contributions of complexes of the first and the second type are approximately equal. The calculated concentrations of free bis-netropsin at different C/BP values were used as the starting data to solve Eqs. [1], [2] and [3] for r_1 and r_2 at different values of K_1, K_2, L_1 and L_2, using Newton's iteration method. The molar dichroism $\Delta\varepsilon$ at different C/BP values was calculated using the experimentally determined values of $\Delta\varepsilon_1$ and $\Delta\varepsilon_2$ at 310 and 340 nm. Then a search was carried out to determine which of the values of binding parameters led to a minimum in the mean square deviation between the measured and calculated values of $\Delta\varepsilon$ at different values of C/BP. Using the best fit values of L_1, L_2, K_1 and K_2, we calculated more precisely concentrations of free bis-netropsin at different C/BP values and, in a second step, calculated r_1, r_2 and $\Delta\varepsilon$. Then a search was carried for a new set of the best fit thermodynamic parameters. Using the CD spectra obtained at two different C/BP values, we also calculated more precisely $\Delta\varepsilon_2$ values at 310 and 340 nm. The process was continued until self-consistent values of binding parameters were obtained. In case of binding to poly[d(AT)]•poly[d(AT)] the best fit between the experimental and theoretically calculated plots of $\Delta\varepsilon$ at 310 nm versus C/BP is observed when $K_1 = 31.6\times10^6$ M^{-1}, $K_2 = 1.0\times10^6$ M^{-1}, $L_1 = 9$ and $L_2 = 4$ (Figure 4, c). The mean square deviation between the measured and calculated values of $\Delta\varepsilon$ is found to be 0.16 $M^{-2}cm^{-2}$. The calculated free ligand concentrations at different C/BP values were used as the starting data for calculation of r_1, r_2 and $\Delta\varepsilon$ at 340 nm. The best fit between the

experimental and calculated plots of $\Delta\varepsilon$ at 340 nm versus C/BP is observed when $K_1 = 2.9\times10^7$ M^{-1} and $K_2 = 3.5\times10^6$ M^{-1}, provided that $L_1 = 9$, $L_2 = 4$ and $\Delta\varepsilon_2 = 22\pm1$ (Figure 4, d). The mean square deviation between the measured and calculated values of $\Delta\varepsilon$ at 340 nm is found to be 0.02 M^{-2} cm^{-2}. Variations in the best fit values of K_1 and K_2 obtained from the CD titrations at 310 and 340 nm might be attributed to the experimental inaccuracies in the evaluation of $\Delta\varepsilon_2$ at 310 and 340 nm.

A similar procedure, with minor modifications, was used to determine thermodynamic parameters for interaction of **Pt-bis-Nt** with duplexes 5'-CC$(TA)_n$ CC-3', where n varies from 4 to 6. Let R_1 and R_2 be the molar ratios of **Pt-bis-Nt** bound in the extended and hairpin form to the 14-mer duplex, respectively. If the ligand binds only to a stretch of 10 consecutive A+T-base pairs in the 14 -mer duplex, then R_1, R_2 and $\Delta D/O$ can be calculated from the following equations:

$$R_1 = 2K_1m / Z \qquad [5]$$

$$R_2 = (7K_2m + 2K_2^2m^2) / Z \qquad [6]$$

$$\Delta D / O = R_1\Delta\varepsilon_1 + R_2\Delta\varepsilon_2 \qquad [7]$$

$$m = C - O(R_1 + R_2) \qquad [8]$$

where $\Delta D/O$ is the measured CD amplitude at a given wavelength expressed per mole of DNA oligomer and 1 cm pathlength cell. O is the molar concentration of DNA oligomer. C is the concentration of the ligand. Z is the grand partition function for the system under study.

$$Z = 1 + 2K_1m + 7K_2m + K_2^2m^2 \qquad [9]$$

The partition function Z is equal to the sum of the statistical weights describing formation of different complexes between **Pt-bis-Nt** and DNA oligomers, including complexes containing a single bound bis-netropsin molecule in the extended confor-mation as well as complexes comprizing one and two bis-netropsin molecules, re-spectively, in the hairpin conformation. The statistical weighting factor for the state when the DNA oligomer contains no bound ligand is assumed to be equal to 1. The numerical coefficient in the second term in Eq. [9] is equal to the number of different placements of a single bis-netropsin molecule in the extended form ($L_1 = 9$) on the DNA oligomer. The third and fourh terms in Eq. [9] represent statistical weights for binding to the DNA oligomer of one and two bis-netropsin molecules, respectively, in the hairpin form ($L_2 = 4$). Eqs.[5]-[9] can be easily modified to describe the binding equilibria in systems containing 12-mer or 16-mer duplexes with the sequences 5'-CC$(TA)_n$ CC-3' where n is equal to 4 or 6.

Figure 8 shows a comparison of the experimental and theoretically calculated plots of $\Delta D/O$ versus C/O for binding of **Pt-bis-Nt** to the 14-mer duplex. The best fit between experiment and theory is observed for $K_1 = 2.9\times10^6$ M^{-1} and $K_2 = 9\times10^5$ M^{-1}. The mean square deviation between the experimental and calculated values of $\Delta\varepsilon$ is found to be 12.1 M^{-2} cm^{-2}. Analysis of the experimental data obtained from titration of the 14-mer duplex by **Pt-bis-Nt** at 340 nm leads to the following values for the best fit parameters: $K_1 = 1.1\times10^6$ M^{-1}, $K_2 = 4\times10^5$ M^{-1}. The mean square deviation between the experimental and

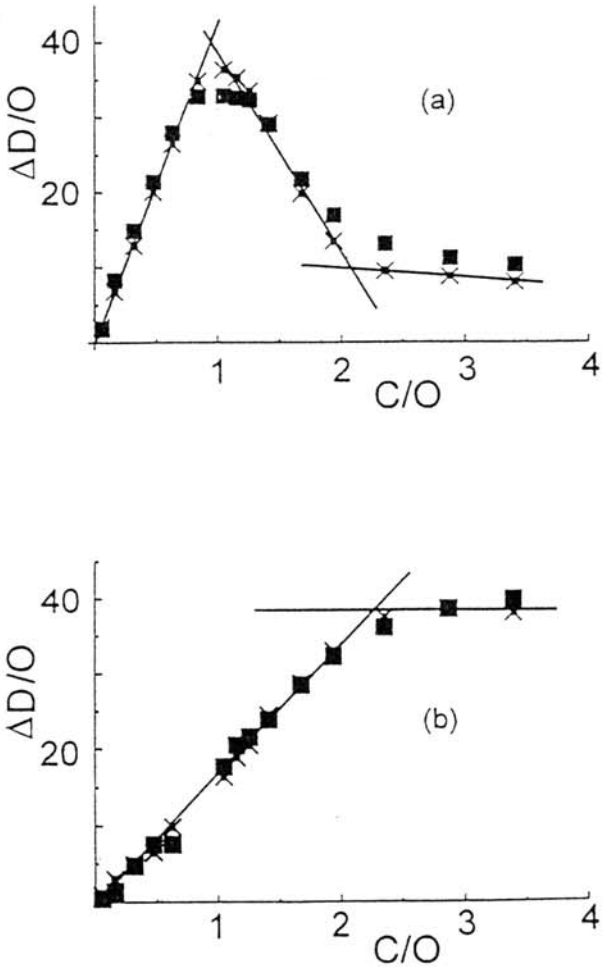


Figure 8: Titrations of the 14-mer duplex (21μM) by **Pt-bis-Nt** at 310 nm (a) and 340 nm (b). ✕, experimental data points; ■, theoretical values of $\Delta D/O$ calculated from Eqs. 5-9 at different C/O values using the best fit values for binding parameters: panel a, $K_1 = 2.9\times10^6$ M^{-1}, $K_2 = 9\times10^5$ M^{-1}; panel b, $K_1 = 1.1\times10^6$ M^{-1}, $K_2 = 4\times10^5$ M^{-1}. Conditions are the same as in Figure 2.

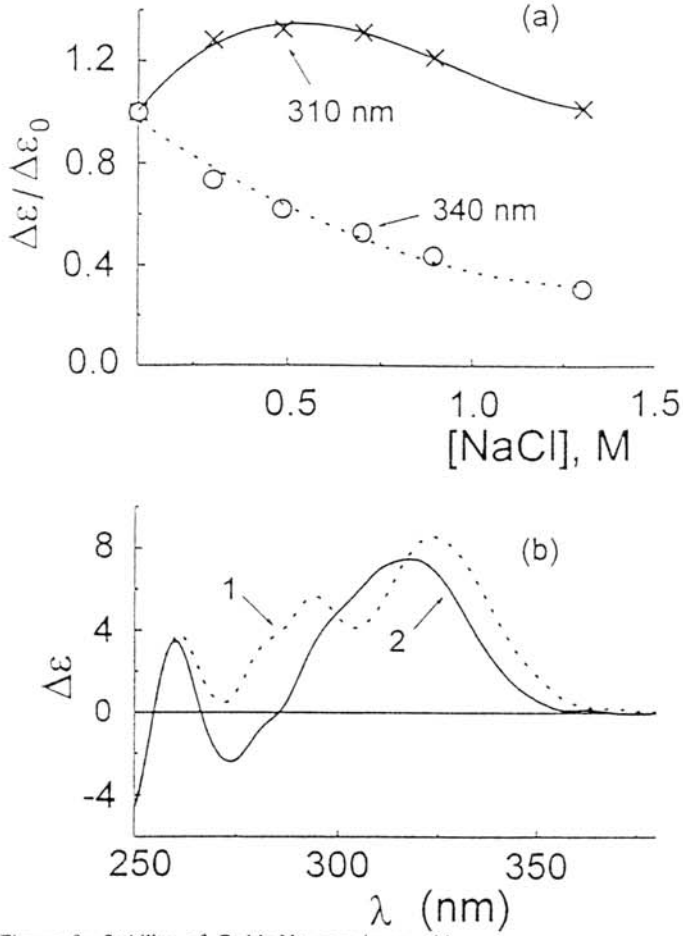


Figure 9: Stability of **Pt-bis-Nt** complexes with poly[d(AT)]•poly[d(AT)] (42 μM base pairs, C/BP=0.38) as a function of NaCl concentration (a). × and o are the experimental data points obtained from measurements of CD amplitude at 310 nm and 340 nm, respectively. (b) CD spectra of complexes between **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)] in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl (curve 1) and 0.6 M NaCl (curve 2).

theoretical values of DD/O is found to be 1.5 M^{-2} cm^{-2}. Comparing the values of K_1 and K_2 obtained from binding of **Pt-bis-Nt** to the 14-mer duplex with those determined for binding to poly[d(AT)]•poly[d(AT)] one can conclude that the ratio of K_2 to K_1 increases in system containing 14-mer duplex approximately by a factor of 10. Thermodynamic analysis of data obtained for binding of **Pt-bis-Nt** to the 12- and 16-mer duplexes shows that decreasing the size of A+T tract in the DNA oligomer leads to an increase of K_2/K_1 ratio (data not shown). An obvious reason for this is that the equilibrium association constant for binding of **Pt-bis-Nt** in the extended conformation decreases in systems contained short DNA oligomers, whereas the affinity constant for interaction of **Pt-bis-Nt** in the hairpin form is found to be less sensitive. This can be attributed to an influence on binding of **Pt-bis-Nt** in the extended conformation of DNA sequences flanking the central A+T- tract or be a consequence of a structural distortion at the duplex ends.

Salt concentration effects on binding of Pt-bis-Nt to poly[d(AT)]•poly[d(AT) and short DNA oligomers

We found that **Pt-bis-Nt** forms more tight complexes with poly[d(AT)]• poly[d(AT)] in the extended conformation than in the hairpin-like form. In Figure 9 and 10, plots of the stability of **Pt-bis-Nt** complexes with poly[d(AT)]•poly[d(AT)] and 14-mer duplex as functions of NaCl concentration are shown. In these experi-ments **Pt-bis-Nt**-DNA complexes were formed in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl, and then the CD amplitudes at 310 nm and 340 nm were measured as functions of the concentration of added NaCl. The results are displayed as plots of the normalized CD amplitude ($\Delta\varepsilon/\Delta\varepsilon_0$) at 310 and 340 nm against the concentration of NaCl in solution. Here $\Delta\varepsilon_0$ denotes the CD amplitude measured at a given wavelength in the presence of 0.1 M NaCl. It is calculated per 1 cm pathlength cell and one mole of DNA base pairs (or one mole of 14-mer duplex in case of binding to the 14-mer duplex). $\Delta\varepsilon$ is the same quantity measured in the presence of added NaCl. As seen from Figures 9, $\Delta\varepsilon/\Delta\varepsilon_0$ at 310 nm is found initially to increase with increase of NaCl concentration in solution until a maximum value is achieved in the presence of 0.6 M NaCl, beyond which $\Delta\varepsilon/\Delta\varepsilon_0$ decreases gradually with further increase of NaCl concentration. The plot of the normalized CD amplitude at 340 nm versus salt concentration suggests that approximately half of the total amount of **Pt-bis-Nt** bound to poly[d(AT)]•poly[d(AT)] in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl dissociates in the presence of 0.6 M NaCl. We interprete these observations as indicating that complex of the second type, which is formed in the presence of 0.1 M NaCl, dissociates at higher salt concentrations and is partially replaced by complex of the first type. Consistent with this interpretation are the CD spectral changes observed for complexes of **Pt-bis-Nt** with poly[d(AT)]•poly[d(AT)] upon increasing salt concentration. In the presence of 0.1 M NaCl the mixture of **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)] (C/BP ≈ 0.4) displays the CD spectrum with two positive CD bands near 290 and 325 nm which are charateristic of the bis-netropsin binding in the hairpin form (Figure 9). Increasing NaCl concentration from 0.1 M to 0.6 M restorted the CD spectrum characteristic of complex the first type between **Pt-bis-Nt** and poly[d(AT)]•poly[d(AT)]. Similar results are obtained for complexes of **Pt-bis-Nt** with the 14-mer duplex (Figure 10).

Concluding Remarks

Two major conclusions can be drawn from our studies on binding of **Gly_3-bis-Nt**

and **Pt-bis-Nt** to poly[d(AT)]•poly[d(AT)] and DNA oligomers. First, these ligands form two types of complexes with poly[d(AT)]•poly[d(AT). The two binding modes can be ascribed to the binding of each bis-netropsin to DNA in the extended and hairpin conformation. Complexes of the first type are characterized by CD spectral profiles which are similar for two bis-netropsins, although sizes of their interaction sites on poly[d(AT)]•poly[d(AT)] are found to be equal to 7 and 9 AT-base pairs for binding of **Gly_3-bis-Nt** and **Pt-bis-Nt**, respectively. Each bis-netropsin binds only in the extended conformation to poly(dA)•poly(dT). Molecular model building studies show that transition of a Pt-bis-netropsin molecule from the hairpin conformation into an extended form can be achieved by rotations around N-C^{α} and C^{α}-C′ bonds in a single glycine residue attached to a platinum (II)-containing linker between two netropsin-like fragments. Second, and this is important for future studies, the CD spectral profiles characteristic of complexes of the second type between poly[d(AT)]•poly[d(AT)] and two bis-netropsins are very different. We suggest that this reflects the fact that **Pt-bis-Nt** and **Gly_3-bis-Nt** use different DNA binding motifs upon binding to poly[d(AT)]•poly[d(AT)] and DNA oligomers in the hairpin forms. Molecular model building studies show that two netropsin-like fragments of Gly_3-bis-netropsin molecule can be inserted into the minor DNA groove using antiparallel side-by-side motif, as it has been observed in the crystalline 2:1 complex between distamycin A and octamer dupelex $d(ICICICIC)_2$ (11). In this complex , the pyrrole rings of one bound antibiotic molecule are stacked on the peptide groups of the other. The hairpin form of **Pt-bis-Nt** involves two parallel strands with the pyrrole rings of one netropsin-like fragment of the bis-netropsin molecule stacking on the pyrrole rings of the other. In the complex , two netropsin-like fragments involved in the parallel motif occupy nonequivalent spatial positions in the minor groove: one fragment forms more favorable contacts with functional groups on DNA base pairs, whereas the other fragment interacts with base pairs less strongly. Different stacking interactions and local environments of two netropsin-like fragments bridged by different linkers and combined in the side-by-side parallel and antiparallel manners in the minor DNA groove might be responsible for the observed difference in CD patterns for complexes of **Pt-bis-Nt** and **Gly_3-bis-Nt** with poly[d(AT)]•poly[d(AT)]. A new parallel side-by-side peptide motif can be used for the design and synthesis of a new generation of sequence-specific DNA-binding ligands.

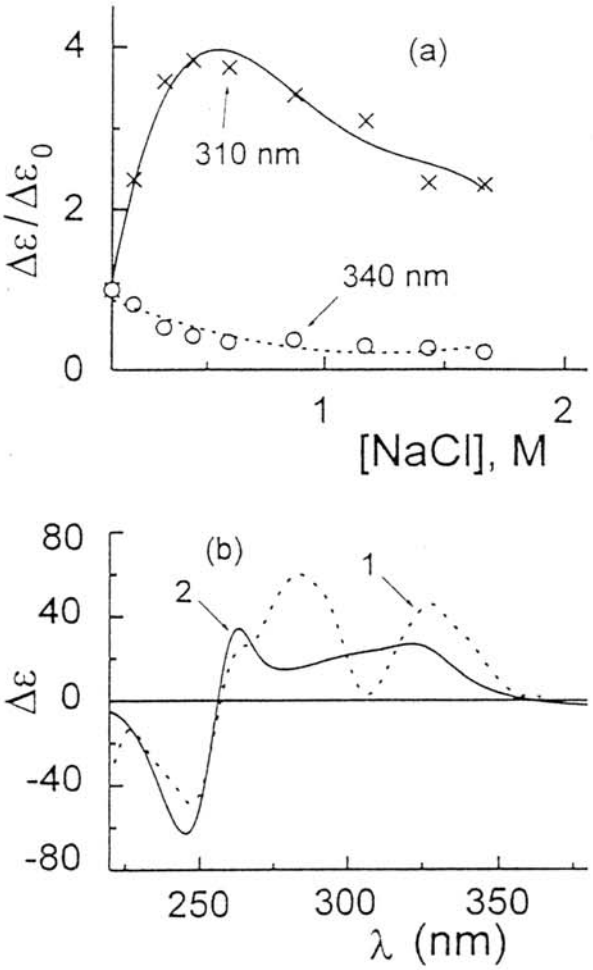


Figure 10: Stability of **Pt-bis-Nt** complexes with 14-mer duplex (24 μM, C/O = 3.2) as a function of NaCl concentration (a). Symbols used are identical to those of Figure 9. (b) CD spectra of complexes between **Pt-bis-Nt** and 14-mer duplex in 1 mM sodium cacodylate buffer (pH 7.0) in the presence of 0.1 M NaCl (curve 1) and 0.8 M NaCl (curve 2).

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