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MOLECULAR BIOPHYSICS

DNA-Binding Activity of Bis-Netropsin Containing a *cis*-Diaminoplatinum Group between Two Netropsin Fragments

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Abstract—The binding of Pt-bis-Nt and its modified analog Pt*-bis-Nt, which has two additional glycine residues in the linker between two netropsin fragments, to DNA has been studied. The elongation of the linker in the bis-netropsin molecule increases the cytotoxicity and leads to an almost complete loss of the antiherpetic activity of bis-netropsin. The study of the binding of two bis-netropsins to an oligonucleotide duplex containing an AT cluster, which is present at the origin of replication of herpes virus (OriS), revealed significant structural differences between the complexes of bis-netropsins with this DNA oligomer. It was shown by CD spectroscopy that the binding of Pt-bis-Nt in the extended conformation and in hairpin form with the parallel orientation of two bis-netropsin fragments makes a greater contribution to the interaction with the duplex than in the case of Pt*-bis-Nt. At high binding levels, Pt*-bis-Nt binds to the AT cluster in OriS predominantly in the form of associates based on the antiparallel, double-stranded, pyrrolcarboxyamide motif. The interaction of Pt-bis-Nt and Pt*-bis-Nt with a single-stranded oligonuclotide (64 nt) corresponding to the upper strand at the origin of replication of herpes virus (OriS*) was also studied. Substantial differences in the binding of bis-netropsins to OriS* and the thermostability of the resulting complexes were found by CD spectroscopy and UV melting studies.

Key words: bis-netropsin, specific recognition in the DNA minor groove, circular dichroism, melting curve, antiherpetic activity

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INTRODUCTION

The design and synthesis of specific DNA-binding compounds capable of selectively binding to the functionally important regions of viral DNA and effective suppressing the viral reproduction are of undoubted interest. The most important results were obtained while using analogs of the anticancer antibiotics netropsin and distamycin as building blocks for the synthesis of new ligands. It was shown by X-ray analysis [1–3] and NMR spectroscopy [4-5] that these antibiotics bind to the clusters of four to five AT pairs in the minor groove of DNA. An obvious way to enhance the selectivity in the binding of these compounds to DNA is the synthesis of dimer (oligomer) derivatives of netropsin and distamycin, which contain two or more monomer fragments covalently connected by different linkers [6–9].

De Clercq and Lown studied the antiviral activity of bis-netropsins where monomer units were covalently linked in the tail-to tail orientation via aliphatic linkers of different length [10]. It was found that bis-netropsins efficiently suppressed the variolovaccine virus reproduction while their antiherpetic activity was revealed at subtoxic concentrations.

In our previous works, a new set of bis-netropsins with the different orientation of the netropsin fragments linked by various linkers was synthesized and investigated [11–13]. Some of these compounds were shown to have significantly higher antiherpetic activity and lower cytotoxicity than netropsin and distamycin or compounds studied by De Clercq and Lown [10–13].

We discovered earlier that small changes in the bis-netropsin structure can significantly influence the equilibrium between various types of the complexes formed by bis-netropsin and DNA. Bis-netropsins can bind to the clusters of AT pairs in the extended conformation occupying approximately one helical turn of DNA. Bis-netropsins in the hairpin form with the parallel and antiparallel orientation of the netropsin fragments can bind to the minor groove of DNA and occupy four to five base pairs; furthermore, they can also form a dimeric complex due to the coalescence of the halves of two bis-netropsin molecules bound at the neighboring overlapping binding sites. All of these complexes can be easily discriminated using CD spectroscopy [14–16]. It was also found that the local DNA structure influenced the affinity and specificity in the binding of different bis-netropsins with DNA [16].

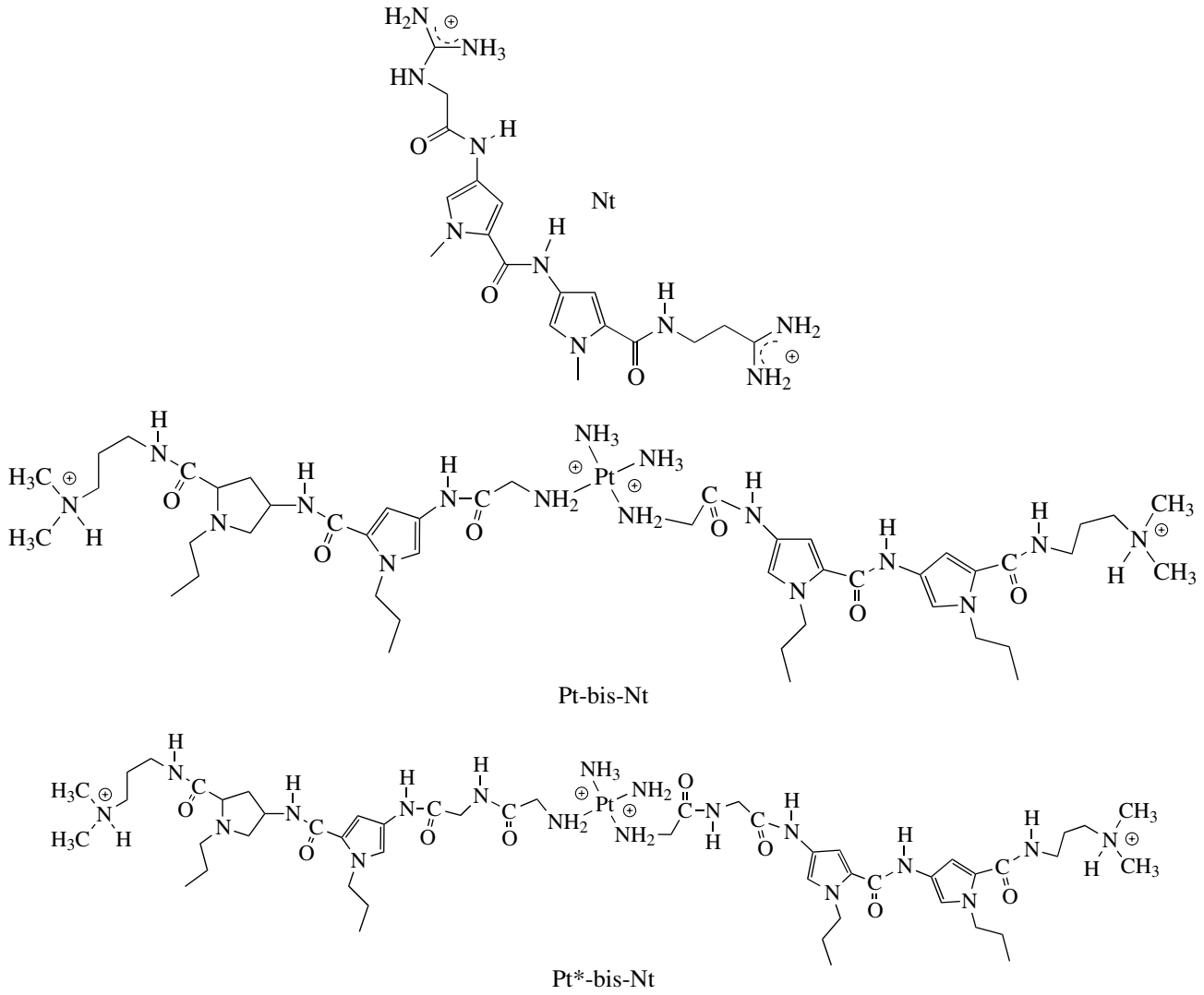

Fig. 1. Chemical formulas of netropsin (Nt) and bis-netropsins, Pt-bis-Nt and Pt*-bis-Nt containing *cis*-diamineplatinum group in the linker.

In our previous work [17], we showed that Pt-bis-Nt and its modified analog Pt*-bis-Nt bound to poly(dA) · poly(dT) using two netropsin fragments predominantly in the extended conformation. It was demonstrated by DNAse footprinting that Pt-bis-Nt bound to the AT-cluster at the origin of replication of herpes virus (OriS) protected a more extended region and had a higher affinity to the AT cluster than Pt*-bis-Nt [17].

In the present work, using CD spectroscopy and other physicochemical methods, we revealed essential differences in the structure of complexes formed by the interaction of bis-netropsins with a duplex containing an AT cluster (18 AT base pairs) located at the site of the initiation of replication of the herpes virus (OriS). We also studied the interaction of Pt-bis-Nt and Pt*-bis-Nt with a single-stranded oligonucleotide (64 nt) that corresponded to the upper strand at the origin of replication of herpes virus (OriS*) and revealed substantial differences in both the DNA binding activity of two bis-netropsins and the thermostability of complexes formed by bis-netropsins with hairpins in OriS*.

EXPERIMENTAL

Ligands. Figure 1 represents the chemical structures of netropsin and two bis-netropsins, Pt-bis-Nt and Pt*-bis-Nt, which contain the *cis*-diamineplatinum group in the linker between two netropsin fragments. The difference of each di-*N*-propylpirrolcarboxamide fragment of the bis-netropsin molecule from netropsin is in the replacement of the C-terminal amidine group, guanidyl acetic acid residue, and *N*-methylpirrolcycles by the tertiary amine residue, glycine residues, and *N*-propylpirrolcycles, respectively. These changes increase the stability of the compounds to degradation in water solutions and probably enhance their ability to penetrate

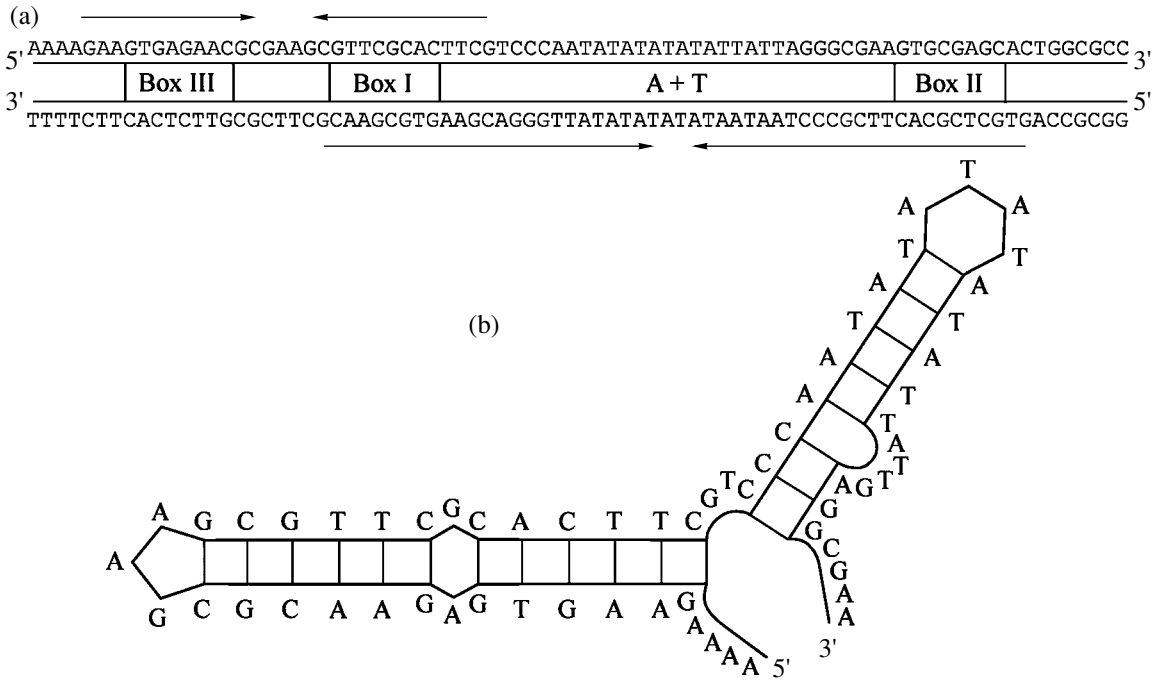

Fig. 2. Nucleotide sequence of the origin of replication of herpes simplex virus I (OriS) (a). Boxes I–III are the binding sites for initiator protein (UL9); palindromic sequences are underlined. The spontaneous secondary structure of the upper strand after unwinding the duplex (b).

through a cell membrane. Pt-bis-Nt and Pt*-bis-Nt were synthesized according to the procedure described in [18]. Concentrations of bis-netropsins were determined by spectrophotometry using the molar absorption coefficient at 297 nm (42000 M^{-1} cm^{-1}).

Oligonucleotide duplex. Oligonucleotides CCA ATA TAT ATA TAT TAT TAG GC (a) and GCC TAA TAA TAT ATA TAT ATT GG (b) were synthesized on an automated synthesizer (Applied Biosystems) using phosphoroamidite chemistry. The molar concentrations of oligonuclotides were determined at 90°C by spectrophotometry using the molar absorption coefficients at 260 nm (267200 M^{-1} cm^{-1} and 271600 M^{-1} cm^{-1} for oligonucleotides *a* and *b*, respectively). In order to form a duplex containing oligonucleotides *a* and *b*, the strands were mixed in equimolar concentrations and the mixture was annealed at 90°C, after which it was slowly cooled to room temperature for 8–10 h. The melting curves of the duplexes have only one transition between the double-stranded and melted states.

The more extended single-stranded oligomer (64 nt) containing the AT cluster and the binding sites for initiator protein UL9 of herpes virus corresponds to the upper strand at the origin of replication of herpes virus (OriS*) (Fig. 2). Before the experiment, oligonucleotide was annealed in 0.01 M cacodylate buffer (pH 7.0) in the presence of 0.1 M LiCl at 97°C for 5 min. The mixture was then immediately placed in ice to enhance the probability of the formation of a hairpin conformation of oligonucleotides.

The CD spectra were registered on a Jasco-720 instrument using cuvettes with optical-path lengths of 1.0, 0.1, and 0.2 cm. The absorption spectra were registered on Jasco V-550 spectrophotometer. Experiments were carried out in 0.01 M Na-cacodylate buffer in the presence of 0.1 M NaCl, pH 7.0.

Bis-netropsins Pt-bis-Nt and Pt*-bis-Nt were dissolved in a small volume of ethanol then added to the above buffer.

RESULTS AND DISCUSSION

Binding of Pt-bis-Nt and Pt*-bis-Nt to 23-mer duplex containing the AT cluster at the origin of replication of viral DNA (OriS). We compared DNA-binding activity of two bis-netropsins towards 23-mer duplex CCA ATA TAT ATA TAT TAT TAG GC/GCC TAA TAA TAT ATA TAT ATT GG. This duplex contains the AT cluster presented at the origin of replication of the herpes virus (OriS) (Fig. 2).

The binding of bis-netropsins to an oligonucleotide can be registered by CD spectra in the region of 300–350 nm. The differential CD spectra for the complexes of Pt-bis-Nt and Pt*-bis-Nt with 23-mer duplex are depicted in Fig. 3. Figure 4 presents the titration curves of the 23-mer duplex by bis-netropsins at 310 nm and 340 nm. The shapes of CD spectra and titration curves indicate the formation of several types of the com-

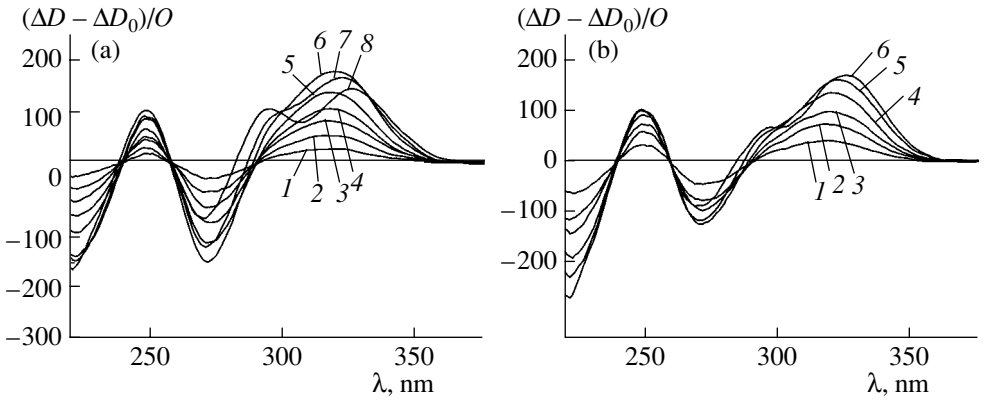

Fig. 3. Differential CD spectra for complexes of Pt-bis-Nt (a) and Pt*-bis-Nt (b) with 23-mer duplex (3 μM). ΔD and ΔD_0 are CD amplitudes calculated for 1-cm optical way in the presence and in the absence of bis-netropsin. *C* and *O* are the molar concentrations of bis-netropsin and the duplex, respectively. *C*/*O*: (a) *1*, 0.25; *2*, 0.50; *3*, 0.74; *4*, 0.99; *5*, 1.32; *6*, 2.07; *7*, 2.50; *8*, 3.31; (b) *1*, 0.47; *2*, 0.95; *3*, 1.26; *4*, 2.00; *5*, 3.15; *6*, 6.30.

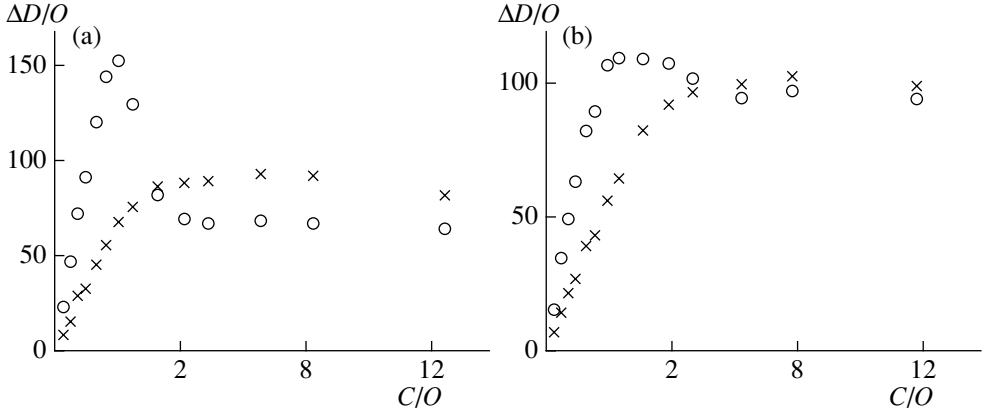

Fig. 4. Titration of the 23-mer duplex (3 μM) with Pt-bis-Nt (a) and Pt*-bis-Nt (b) at 310 nm (circles) and 340 nm (crosses).

plexes. At low bis-netropsin occupancy of the duplex ($C/O < 2$, where *C* and *O* are the molar concentrations of bis-netropsin and the duplex respectively) CD spectra of the complexes coincided with each other and were similar to CD spectra of complexes of bis-netropsins with poly(dA) · poly(dT). At low binding levels, both bis-netropsins are attached to DNA via two netropsin fragments in the extended conformation. The spectral differences are observed at the high ratio of each bis-netropsin to the 23-mer duplex ($C/O > 3$). In this case, complexes with Pt-bis-Nt have the characteristic CD spectrum containing two maximums at 295 nm and 330 nm. As was shown previously [14–16], the presence of two positive CD bands at these wavelengths indicates that bis-netropsin binds to DNA in the hairpin form with the parallel orientation of two netropsin fragments. In case of the binding of Pt*-bis-Nt to the 23-mer duplex, the shift in the positive long wavelength maximum from 315 nm to 325 nm and the shoulder at 295 nm were observed, which are characteristic to the binding of the netropsin fragments in sandwich form with their antiparallel orientation [14]. The titration curves at 310 nm and 340 nm (Fig. 4) have a more complicated form compared to those for binding Pt-bis-Nt to poy(dA) · poly(dT) [14]. The CD amplitude at 310 nm for the complexes of Pt-bis-Nt with the 23-mer duplex decreases at $C/O > 2$, which indicates the binding of bis-netropsin in the hairpin form. As for the CD spectra of the complexes of Pt*-bis-Nt with this duplex, only the insignificant decrease of the CD amplitude is observed in the range of the bis-netropsin loading $2 < C/O < 3$.

The differential CD spectra of the complexes of Pt-bis-Nt and Pt*-bis-Nt with OriS DNA fragment from herpes virus can be presented as the superposition of three spectra corresponding to the different types of the complexes of bis-netropsin with oligomer DNA. It is assumed that bis-netropsins can be attached to DNA in a completely extended conformation via two netropsin fragments, in hairpin form with the parallel orientation of two netropsin fragments, and in the form of associates stabilized by the stacking between the netropsin fragments of two or three molecules of bis-netropsin.

The Pt-bis-Nt and Pt*-bis-Nt molecules in the extended conformation bind to eight and nine base pairs of DNA, respectively, while in parallel hairpin form, each bis-netropsin occupies five base pairs. The CD spectra for binding bis-netropsin in the extended conformation and in parallel hairpin form can be calculated from the experimental CD spectra for the complexes of bis-netropsin with poly[d(AT)] · poly[d(AT)] and DNA oligomer 5'-CGTATATCG-3' [14–16]. It was found previously that Pt-bis-Nt bound to the latter duplex in hairpin form with the parallel orientation of two netropsin fragments in the minor groove of DNA. We also previously obtained important results concerning the CD spectra for the complexes of the other bis-netropsin with the DNA oligomer with the sequence 5'-CGTATATACG-3', which may indicate the binding of bis-netropsins in the associate form. The netropsin fragments in this bis-netropsin were covalently linked in head-to-tail orientation by the triglycine residue, so that bis-netropsin could not bind to the duplex in the extended conformation (the AT cluster contained only six base pairs) or in hairpin form with a parallel orientation of two netropsin fragments. This bis-netropsin formed the complex with the oligomer with a stoichiometry of 2 : 1. The differential CD spectrum of this complex indicates the binding of the netropsin fragments of two bis-netropsin molecules in the sandwich form to the minor groove of DNA. Two other netropsin fragments in this dimer complex were close to the GC pairs being, apparently, unbound. There is another situation upon the binding of Pt-bis-Nt and Pt*-bis-Nt in the dimer form to the extended AT cluster in OriS when both the formation of two-stranded pirrolcarboxamide motif in the minor groove of DNA and the binding of the single-stranded netropsin fragments take place.

Let $\Delta\varepsilon_1(\lambda)$ be the molar dichroism of the netropsin fragment at wavelength λ. If the netropsin fragments make additive contributions in the CD amplitude of the complex, the molar dichroism of bis-netropsin in the extended conformation is $2\Delta\varepsilon_1(\lambda)$. Let $\Delta\varepsilon_2(\lambda)$ be the molar dichroism of the bis-netropsin bound in the hairpin form with the parallel orientation of two netropsin fragments and $\Delta\varepsilon_3(\lambda)$ be the molar dichroism of a sandwich consisting of two netropsin fragments in antiparallel orientation. The molar dichroism of the associate consisting of two linked bis-netropsin molecules is $2\Delta\varepsilon_1(\lambda) + \Delta\varepsilon_3(\lambda)$ and the molar dichroism of the trimeric complex is $2\Delta\varepsilon_1(\lambda) + 2\Delta\varepsilon_3(\lambda)$. Let R_1 and R_2 be the molar ratios of bis-netropsin (per one mole of the oligomer) bound to DNA in an extended conformation and hairpin form with the parallel orientation of the two netropsin fragments, respectively. Let R_3 and R_4 be the molar ratios of the bound dimers and trimers of bis-netropsin. The differential CD spectra of the complexes of bis-netropsin with the DNA oligomer can be presented as the following superpositions of the differential CD spectra mentioned above:

$$(\Delta D - \Delta D_0)/O = 2R_1\Delta\varepsilon_1(\lambda) + R_2\Delta\varepsilon_2(\lambda) + R_3(\Delta\varepsilon_3(\lambda) + 2\Delta\varepsilon_1(\lambda)) + R_4(2\Delta\varepsilon_1(\lambda) + 2\Delta\varepsilon_3(\lambda)), \quad (1)$$

$$m = C - O(R_1 + R_2 + 2R_3 + 3R_4), \quad (2)$$

where O, C, and m are the molar concentrations of the DNA oligomer, bis-netropsin in solution, and free bis-netropsin, respectively; ΔD and ΔD_0 are the CD amplitudes of the complex of DNA with bis-netropsin and free DNA, respectively, calculated for a 1-cm optical path. Decomposition of the differential CD spectra into components allows one to determine the relative content of complexes of different types. To simplify the calculations, we optimized the differential CD spectra typical for the binding of bis-netropsins in the associate form. The comparison of the calculated and the expected CD spectra for the dimer and trimer complexes showed that the optimization corresponded to an average spectrum of dimer and trimer complexes. The resulted spectrum provided the minimum of the mean square deviation between the experimental and calculated CD spectra. The optimized spectrum appeared to be the same for complexes of DNA with Pt-bis-Nt and Pt*-bis-Nt.

The above-mentioned spectra were used for the decomposition of the differential CD spectra into components (Fig. 5); they characterize the binding of bis-netropsins in extended conformation, parallel hairpin form, and in the form of the antiparallel dimer. The experimental and calculated CD spectra for the binding of Pt-bis-Nt and Pt*-bis-Nt to the 23-mer duplex at $C/O = 3$ are depicted in Fig. 6. The calculated values of R_1, R_2, and R_3 for the complexes of Pt-bis-Nt (a) and Pt*-bis-Nt (b) with the 23-mer duplex (Fig. 7) indicate that Pt-bis-Nt in the extended conformation and in the hairpin form makes the higher contribution to the binding than Pt*-bis-Nt at the similar C/O ratios. Unlike Pt-bis-Nt, Pt*-bis-Nt has a greater tendency to bind to DNA in associate form (dimer, trimer) based on the double-stranded antiparallel pirrolcarboxamide motif. In the range of C/O of 2–5, the contribution to the binding of Pt-bis-Nt in hairpin form is higher by a factor of approximately five than that of Pt*-bis-Nt in the same form.

Interaction of Pt-bis-Nt and Pt*-bis-Nt with the single-stranded oligonucleotide OriS*. It is known [19–22] that during replication of the viral DNA, initiator protein UL9 of herpes virus selectively binds to the origin of replication of the viral DNA (OriS) and, in the presence of ATP and another ICP8 viral protein, completely unwinds the minimal duplex (80 bp) and forms the stable complex only with the upper strand of this duplex (OriS*). In the absence of the protein, OriS* is spontaneously folded into two hairpins (Fig. 2). One of them is formed due to the complementary pairing of nucleotides located in boxes I and III. The second hairpin contains five AT base pairs formed by nucleotides presented in the AT cluster. This hairpin is not stable

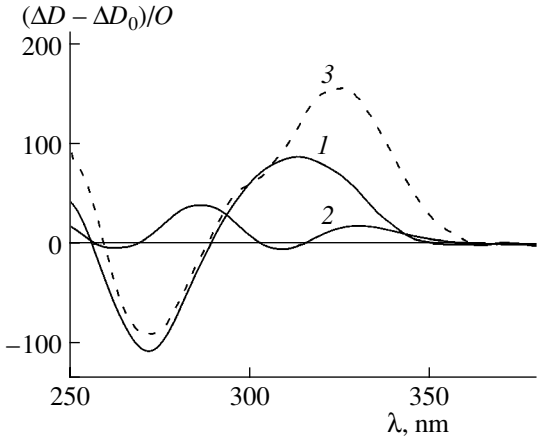

Fig. 5. The referenced spectra used for the decomposition of the differential CD spectra into components and characterizing the binding of bis-netropsins in the extended conformation (*1*), in the form of the parallel hairpin (*2*), and in the form of the associate based on the antiparallel double-stranded pirrolcarboxamide motif (*3*).

enough and unwinds under slightly increasing temperature that results in the formation of the single-stranded fragment at the 3'-end of OriS* oligomer.

We studied the interaction of Pt-bis-Nt and Pt*-bis-Nt with OriS* by CD spectroscopy. One can expect that AT-specific ligands can interact with the short hairpin in OriS* containing five AT base pairs. Stabilization of the hairpin structure in the complex with bis-netropsin can lead to the inhibition of viral DNA replication because the presence of the single-stranded fragment at the 3'-end is necessary to the normal function of UL9 helicase. The CD spectra for the complexes of bis-netropsins with OriS* sufficiently differ by both the amplitude and position of the long wavelength maximum (Fig. 8). The long wavelength maximums in the CD spectra for complexes of oligonucleotide with Pt-bis-Nt and Pt*-bis-Nt are observed at 327–330 nm and 317–320 nm, respectively. The shift in the maximum from 315 nm to 330 nm is an argument in favor of parallel hairpin formation upon the binding of Pt-bis-Nt to OriS*, while the maximum at 317–320 nm for the complex of oligomer with Pt*-bis-Nt indicates the monodentant binding of this compound to the AT-rich hairpin in OriS*, i.e., the binding of one of two netropsin fragments.

The UV melting curves of hairpins formed by OriS* in the absence of bis-netropsins and those of the complexes of OriS* with bis-netropsins are presented in Fig. 9. In the former case, the curve is biphasic, probably due to the difference in thermostability of two hairpins, i.e., via the short AT-rich hairpin melting at low temperature (35°C) and extended GC-rich hairpins formed by the pairing of nucleotides in boxes I and III, which has a much higher T_m (82°C). In the complexes with bis-netropsins, the melting temperature of the short hairpin increases and that of the extended hairpin almost dos not change (Fig. 9 and table). The T_m values of the short hairpin in complexes of OriS* with Pt-bis-Nt and Pt*-bis-Nt increase by 22°C and 12°C, respectively.

Virological experiments showed that the addition of two glycine residues to the linker containing a *cis*-diamineplatinum group led to an increase in the cytotoxicity, as well as a complete loss of the antiherpetic activity of bis-netropsin [17]. The difference in the antiherpetic activity of two bis-netropsins may be due to their different abilities to penetrate the nuclei of eukaryotic cells where the replication of the DNA of the herpes virus takes place. Pt-bis-Nt was previously shown to penetrate the xenopus oocytes nuclei and attach to specific binding sites in DNA in the chromatin context. We believe it is highly unlikely that the addition of two glycine residues to the linker can have a large influence on the ability of bis-netropsins to penetrate the nuclei of eucaryotic cells.

The results of DNase footprinting indicate a difference in the affinity and selectivity of the binding of two bis-netropsins to the AT cluster in OriS [17]. The asso-

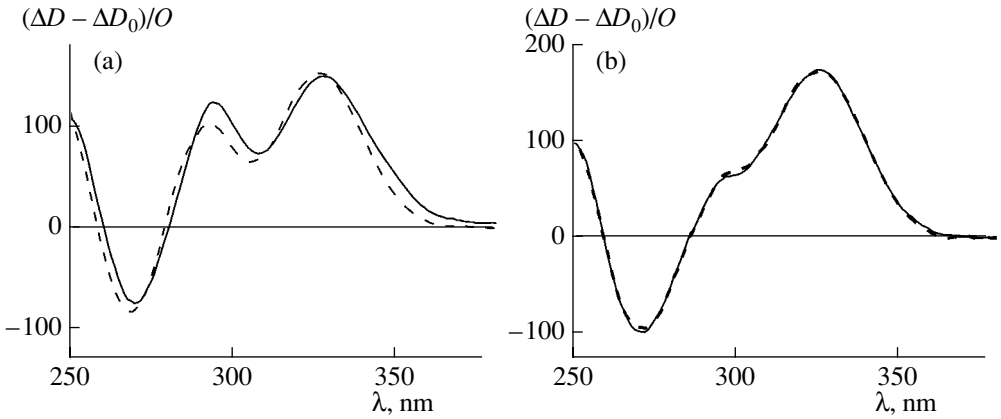

Fig. 6. Experimental and calculated differential CD spectra for the complexes Pt-bis-Nt (a) and Pt*-bis-Nt (b) with 23-mer duplex at $C/O = 3$.

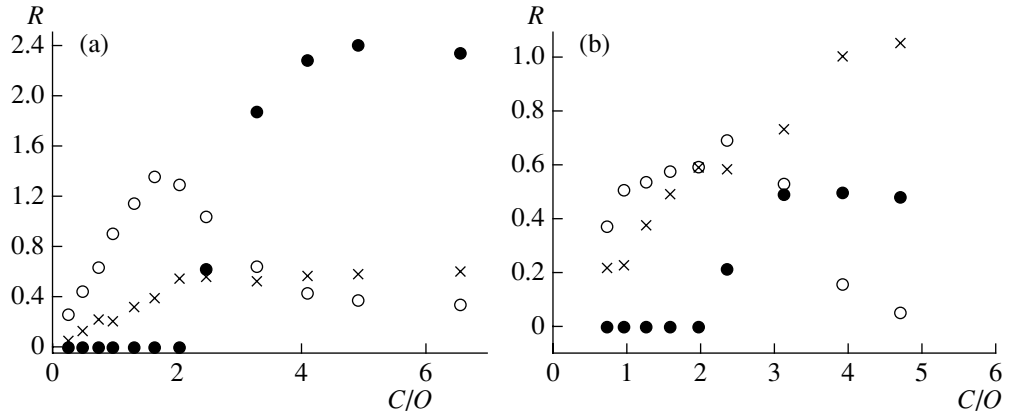

Fig. 7. Comparison of the contributions of the different types of the complexes of Pt-bis-Nt (a) and Pt*-bis-Nt (b) with 23-mer duplex at different values of *C*/*O*. R_1, R_2, and R_3 are the molar ratios of bis-netropsins (per one mole of the duplex) bound to DNA in the extended conformation (white circles), in the hairpin form (dark circles), and the connected associates of bis-netropsin (crosses), respectively.

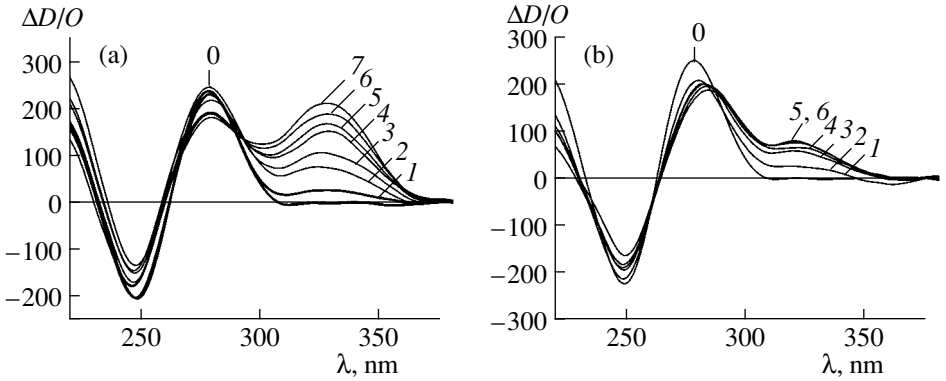

Fig. 8. CD spectra of the complexes of Pt-bis-Nt (a) and Pt*-bis-Nt (b) with the single-stranded oligonucleotide OriS* presented in the upper strand at the origin of replication of herpes virus (0.6 μM) at different ratios of bis-netropsins to the oligomer. *C*/*O*: (a) *1*, 0.5; *2*, 1.4; *3*, 2.2; *4*, 3.3; *5*, 4.3; *6*, 5.4; *7*, 6.8; (b) *1*, 1.17; *2*, 2.7; *3*, 4.0; *4*, 5.3; *5*, 6.7; *6*, 8.3.

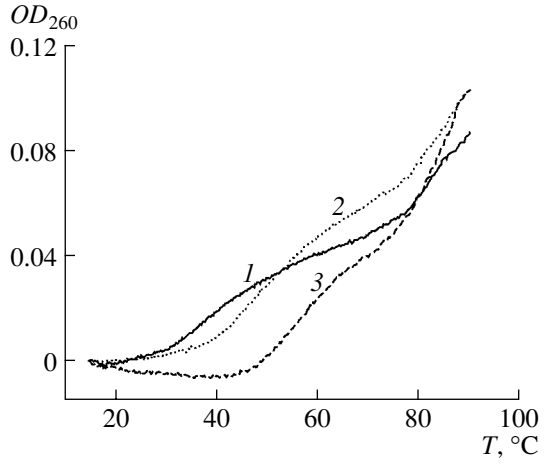

Fig. 9. UV melting curves at 260 nm of OriS* without ligands (*1*), in the complexes with Pt*-bis-Nt (*2*), and with Pt-bis-Nt (*3*).

ciation constant of the 23-mer duplex with Pt-bis-Nt in the extended conformation appeared to be much higher as compared to that with Pt*-bis-Nt [17]. Antiherpetic activity of bis-netropsins may be due to the inhibition of the activity of UL9 helicase of the herpes virus. The binding of bis-netropsins with the AT cluster can suppress the local opening of AT base pairs caused by thermal fluctuations and, thereby, inhibit the destabilization of the AT cluster and the formation of the DNA bending upon the binding to UL9 helicase. Not only the stability, but also the lifetime of the complex of DNA with bis-netropsin is of great importance for the unwinding of DNA by UL9 helicase. It is shown in this work that the structure and the type of the complexes formed by bis-netropsins with the AT cluster essentially vary. Pt-bis-Nt binds to the AT-cluster predominantly in the hairpin form, and the contribution of this form to the binding is higher by approximately a factor of five in comparison with the hairpin binding of Pt*-bis-Nt. We

Table

Compound	T_1, °C	T_2, °C
O_1 (upper strand)	35	82
O_1 + Pt-bis-Nt	57	84
O_1 + Pt*-bis-Nt	47	83

revealed also the sufficient difference in the binding of bis-netropsins to the single-stranded oligonucleotide presented in the upper strand at the origin of replication of the viral DNA (OriS*). In accordance with the works [19–22], this oligonucleotide is spontaneously folded into two hairpins. For the normal action of helicase and the subsequent replisome assembling, the short hairpin containing the cluster of five AT base pairs should be unwinded. We showed the strong difference between the CD spectra of the complexes of OriS* with Pt-bis-Nt and Pt*-bis-Nt (Fig. 8). The thermostability of these complexes is also different; the T_m values for the complex with Pt-bis-Nt and Pt*-bis-Nt increase by 22 and 12°C, respectively (Fig. 9 and table). One may assume that the binding of Pt-bis-Nt in the hairpin form with the parallel orientation of two netropsin fragments to the AT-rich hairpin leads to stabilization of the hairpin and to inhibition of the formation of the single-stranded DNA fragment, which is necessary for the UL9 helicase action. Judging by the CD spectra, Pt*-bis-Nt binds to OriS* in the monodentant manner; it stabilizes the AT-rich hairpin structure to a lesser extent than Pt-bis-Nt. This fact may be correlated to the observed difference in the antiherpetic activity of two bis-netropsins.

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