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DNA structural alterations induced by bis-netropsins modulate human DNA topoisomerase I cleavage activity and poisoning by camptothecin

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Abstract

Bis-netropsins (bis-Nts) are efficient catalytic inhibitors of human DNA topoisomerase I (top I). These DNA minor groove binders are considered to serve as suppressors of top I-linked DNA breaks, which is generally believed to be related to their affinity to DNA. In this study, it was found that bis-Nts exhibit sequence-specificity of suppression of the strong top I-specific DNA cleavage sites and that this sequence-specificity is determined by differential ligand-induced structural alterations of DNA. Raman scattering analysis of bis-Nts interactions with double-stranded oligonucleotides, each containing the site of specific affinity to one of bis-Nts and a distinctly located top I degenerate consensus, demonstrated that bis-Nts induce not only structural changes in duplex DNA at their loading position, but also conformational changes in a distant top I-specific DNA cleavage site. The ability to alter the DNA structure correlates with the anti-top I inhibitory activities of the ligands. In addition, DNA structural alterations induced by bis-Nts were shown to be responsible for modulation of the camptothecin (CPT)-mediated DNA cleavage by top I. This effect is expressed in the bis-Nts-induced enhancement of some of the CPT-dependent DNA cleavage sites as well as in the CPT-induced enhancement of some of the top I-specific DNA cleavage sites suppressed by bis-Nts in the absence of CPT.

Keywords: Topoisomerase I; Netropsin; Camptothecin; Sequence specifcity; DNA cleavage

1. Introduction

Top I alters DNA topology by transiently breaking and rejoining single DNA strands [1]. There is increasingly much evidence that top I plays an important role in DNA replication, transcription and recombination [1,2]. Apart from its physiological functions, top I has been found to be the primary cellular target for various antitumor agents [3]. These agents may be divided into two main groups: top I ''poisons'' and top I ''suppressors.'' Drugs of both types inhibit top I-mediated DNA relaxation. However, top I poisons, but not suppressors, interfere with the breakage-reunion reaction of the enzyme by trapping the reaction intermediate known as a ''cleavable complex'' [2].

The well-known antibiotic Nt and its derivatives bind non-covalently to AT stretches of double-stranded DNA [4] and are capable of inhibiting numerous DNA-binding enzymes, such as bacterial RNA and DNA polymerases and DNase I [4], gyrases [5], mammalian DNA topoisomerases I [6] and II [7], etc. Nts bind to DNA within the minor groove through the hydrogen bonds formed between the nitrogen atoms of their amide groups and O2 atoms of thymines and/or N3 atoms of adenines [8–11]. These data have served as a theoretical basis for synthesis of new Nt analogs, bis-Nts, comprising two Nt moieties linked with a flexible spacer [12–14]. Bis-Nts cover an entire turn of the DNA double helix, with the specificity of their binding being considerably higher than that of Nts binding [12,15]. Recent studies demonstrated that some bis-Nts possess a several-fold higher antitumor and antiviral activity than Nt

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Abbreviations: Top I, human DNA topoisomerase I; Nt(s), netropsin(s); Bis-Nt(s), bis-netropsin(s); CPT, camptothecin; OLIG(s), double-stranded DNA oligomer(s).

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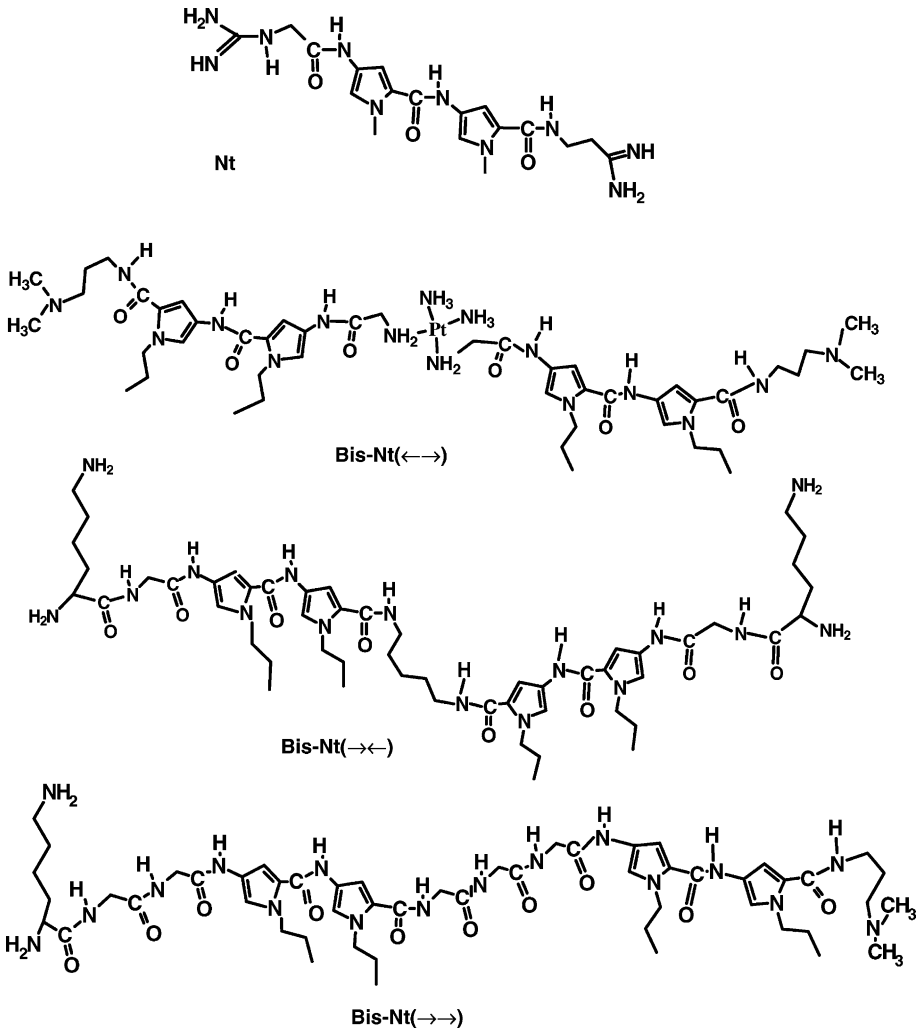


Fig. 1. Chemical structures of Nt and bis-Nts.

due to modulation of activity, or inhibition, of different DNA-binding enzymes [16–18].

We have synthesized a series of sequence-specific bis-Nts [19–21], where the Nt subunits are linked in different orientations. These new bis-Nts are designated as bis-Nt(→←), bis-Nt(←→) and bis-Nt(→→), where arrows indicate the direction of peptide bonds (CO → NH) in the Nt subunits (Fig. 1). Their effects on the DNA cleavage by top I have been described in our recent studies. There are: (i) disappearance of some of the top I-specific DNA cleavage sites; (ii) enhancement of some other sites; and (iii) induction of new DNA cleavage sites [18]. Because top I alone is not the only target of bis-Nts, all these findings require detailed analysis of the structural alterations induced by these ligands on the DNA level upon their binding.

In this study we employed spectroscopic and biochemical techniques to evaluate the structural effects of bis-Nts binding to DNA. First, we analyzed binding of bis-Nt(←→), bis-Nt(→←), and bis-Nt(→→) to three 20-meric DNA duplexes by means of the Raman spectroscopy. Each duplex contained a sequence whose binding with one of the ligands studied was characterized by a binding constant at least 10-fold higher compared with its binding with other bis-Nts [19–22] and a structurally distinct sequence corresponding to the top I degenerate consensus, which was the same in all of the three duplexes. Second, we used the DNA fragment containing these duplexes as a top I substrate and estimated the capacities of different bis-Nts for affecting the enzyme-mediated cleavage in the absence or presence of the top I poison CPT. We found a correlation between the ability of bis-Nts to alter the structural parameters of duplex DNA and their ability to inhibit top I. Moreover, these structural effects were shown to be responsible for bis-Nts modulation of CPT-induced DNA cleavage by top I.

In total, the data obtained are the first experimental evidence that bis-Nts modulate the top I-mediated DNA cleavage in a sequence-selective manner both in the absence and in the presence of CPT and that this sequence-selectivity is determined by differential DNA structural alterations caused by ligand binding.

2. Materials and methods

2.1. Chemicals and enzymes

Bis-Nt(←→) was synthesized as described in [19], and bis-Nt(→←) and bis-Nt(→→) were synthesized as described in [21]. CPT was from Sigma. Plasmid pGEM-7Z(f+) and restriction endonucleases were from Promega and the *E. coli* strain ''Sure'' was from Stratagene. Bovine pancreatic DNase I and Klenow fragment of *E. coli* DNA polymerase I were from Sigma and Boehringer, respectively. Recombinant 68 kDa top I was purified to homogeneity from insect cells using a two-step procedure and was shown to have the same catalytical activities as the full-length enzyme [23]. The specific activity of the 68 kDa top I used in our assays was 1.8×10^6 units/mg (one unit of activity was defined as the amount of enzyme yielding 100% of relaxation of 300 ng of supercoiled pGEM-7Z(f+) plasmid DNA at 37° within 30 min).

2.2. Synthetic DNA substrates

The oligomers 5′-CAAAGACTTAGTTTTAAAAA-3′ (1); 5′-TTTTTAAAACTAAGTCTTTG-3′ (2); 5′-CAAAGACTTAGAAAATTTTT-3′ (3); 5′-AAAAATTTTCTAAGTCTTTG-3′ (4); and 5′-CAAAGACTTAGAAAAAAAAA-3′ (5); 5′-TTTTTTTTTCTAAGTCTTTG-3′ (6) were synthesized by Eurogentec (Belgium) and purified by means of PAGE. The double-stranded DNA oligomer(s) (OLIG(s)) (1) + (2), (3) + (4), and (5) + (6) were prepared by mixing equal molar quantities of two complementary strands in water. These mixtures were annealed at 95° for 15 min and cooled to room temperature overnight. Then the solutions were evaporated to dryness and dissolved in a TE buffer (10 mM Tris–HCl, pH 8.0 and 1 mM EDTA) to the final concentration of 1.2 mM. Ligand–OLIG complexes were obtained by mixing equal molar quantities of bis-Nt and OLIG. OLIG1—(1 + 2), OLIG2—(3 + 4), and OLIG3—(5 + 6), were regarded as ''specific'' with respect to the binding of bis-Nt(←→), bis-Nt(→←), and bis-Nt(→→), respectively, and ''non-specific'' with respect to the other two bis-Nt, based on the fact that the binding constants of bis-Nt(←→) with OLIG1, bis-Nt(→←) with OLIG2, and bis-Nt(→→) with OLIG3 (specific binding) were at least 10-fold higher than the constants of their binding with the each of the other two OLIGs (non-specific binding) [19–22].

2.3. Preparation of the top I–DNA substrate

The top I–DNA substrate was prepared in the form of a plasmid construct containing a number of specific top I cleavage sites as described in [18]. The end-labeled DNA fragment was obtained by cleaving the plasmid with *Xba*I and *Mlu*I. In order to introduce a radioactive label at one of the 3′-ends of the fragment [^{33}P]dATP, non-labeled other dNTPs, and Klenow fragment of *E. coli* DNA polymerase I were used, with subsequent separation of DNA fragments on non-denaturing 5% (w/v) PAGE.

2.4. Top I-mediated cleavage

DNA cleavage by top I was carried out by incubating 50 units of top I with radioactively labeled DNA fragment(3,000–10,000 cpm) in 10 mM Tris–HCl (pH 7.8), 5% glycerol, 0.25 mM EDTA, and 0.3 mM 2-ME (final volume, 20 μL). For the DNA cleavage in the presence of drugs, 10 μM of CPT and/or different concentrations of bis-Nts were used. Reactions mixtures were incubated at 25° for 30 min, then SDS and proteinase K were added to concentrations of 0.5% (w/v) and 1 mg/mL, respectively. After additional incubation for 60 min at 37°, DNA was purified by extraction with phenol, precipitated with ethanol, washed with 70% ethanol, and dried.

2.5. Gel electrophoresis

The samples of DNA were dissolved in 1.5 μL of a formamide–dye mixture (90% formamide containing 15 mM EDTA (pH 8), 0.05% bromphenol blue, and 0.05% xylene cyanole FF dyes); heated for 1 min at 90°; cooled to 0°; and applied on to a 40 cm long 8% denaturing PAGE with a gradient thickness of 0.15–0.45 mm. Electrophoresis was conducted for 65 min at 65 W (2500 V). Before exposure, the gel was fixed with 10% acetic acid and dried on a glass pretreated with Bind-silane (LKB). Cleavage products were identified by means of comparison with the ''A + G'' Maxam–Gilbert sequencing ladder. The rate of the top I-mediated DNA cleavage was determined by densitometry analysis using BioRad software.

2.6. Raman spectroscopy

Raman spectra were recorded with a Ramanor HG-2S (Jobin Yvon) spectrometer; 514.5 nm line of an argon laser (Spectra Physics, model 164-03) was used for excitation. The irradiation power on the sample did not exceed 200 mW and all spectra were recorded with scanning at a step of 1 cm^{-1} and an integration time of each point of 3 s. For Raman spectra recording, pooled data on 10 independent scans averaged with respect to time were used. The stability of the samples during the laser irradiation was controlled by comparison of the spectra as a function of time. The spectra of free (unbound) bis-Nts and OLIGs were obtained by subtracting the spectrum of the buffer from the corresponding bis-Nt/buffer and OLIG/buffer spectra. The same procedure was used to obtain the spectra of bis-Nt/OLIG complexes. The standard buffer spectrum was subtracted from the experimental spectra using the water band at 1645 cm^{-1} as an internal control. The spectra were treated using the LabCalc (Galactic Industries) software as described in [23].

3. Results

3.1. The effect of bis-Nts on the formation of top I-linked DNA breaks

The top I–DNA intermediate cleavable complex contains an enzyme-mediated DNA single break. In fact, the exact position of this break may be determined by separation of radiolabeled products of the top I-mediated DNA cleavage by means of denaturing gel electrophoresis after treatment with SDS and proteinase K [24]. Autoradiography followed by comparative densitometric analysis of the intensities and distribution of cleavage bands provides information on top I preferential recognition of its DNA substrate and the covalent complex formation.

In the present work, we studied the effects of different bis-Nts on top I recognition and cleavage of the restricted fragment of a model DNA construct. This construct had a characteristic distribution of the DNA consensus sequences cleaved by top I within an approximately 200 bp of the polylinker region of plasmid pGEM-7Z(f+) (Fig. 2). The upstream regions of the DNA cleavage sites A–D (Fig. 2) were similar, whereas the downstream sequences corresponded to the high-affinity binding sites of bis-Nts(←→), bis-Nts(→←) and bis-Nts(→→). These sequences, differing in the order of adenine and thymine tracts (Fig. 2), were located at the positions from +3 to +10 relative to the cleavage sites.

Fig. 2 shows that, in the absence of drugs, four strong A–D top I-induced DNA cleavage sites are revealed. Top I cleaves DNA at the sites B, C, and D at about equal rates and more efficiently than at the site A. Sites B and C contain the homo-AT tracts A_8 and A_4, respectively, downstream of top the I cleavage sites. The downstream region of the site D contains the A_4T_4 tract, and its complete sequence is highly homologous to the well-known top I high-affinity DNA-binding site in *Tetrahymena thermophila* extrachromosomal rDNA [25]. Site A includes an inverted, relative to site D, sequence in its downstream region (T_4A_4). It should be noted that the inversion of this downstream sequence provokes a strong reduction in the rate of DNA cleavage by top I (Fig. 2).

All of the three synthetic bis-Nts used in this work exhibited strong top I inhibitory activities [18]. Moreover, the use of this model DNA substrate in the top I cleavage assay showed a sequence-selective inhibition of cleavage by different bis-Nts used at the same (0.2–25 μM) concentrations (0.2–25 μM). Fig. 2 shows that bis-Nt(→→) has a negligible effect on the DNA cleavage by top I at the all top I-specific sites of the DNA fragment as compared to bis-Nts(→←) and bis-Nt(←→). Otherwise, the effects of bis-Nts on the top I-mediated DNA cleavage at the sites A, B and D were found to be different: the same concentrations of drugs suppressed the DNA cleavage at some sites stronger than at others (Fig. 2).

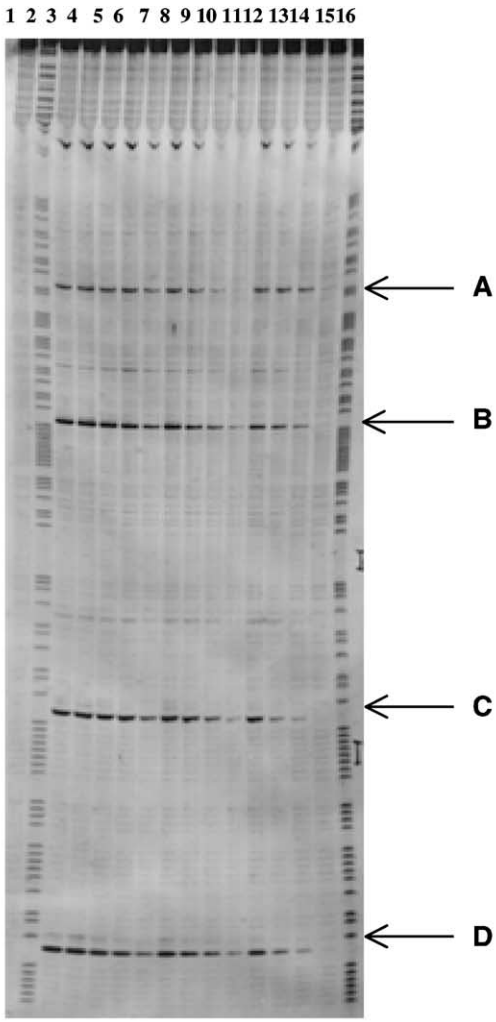
Fig. 3 shows the results of comparative densitometric analysis of DNA cleavage rates at the sites A, B, and D as dependent on the concentrations of the ligands. Under the assay conditions, non-symmetrical bis-Nt(→→) reduces the cleavage at the site A stronger than at the site D and did not affect it at the site B. The rate of DNA cleavage inhibition by symmetrical bis-Nts was found decreasing in the following order: A > D > B and D > B > A for bis-Nt(→←) and bis-Nt(←→), respectively.

3.2. The effects of bis-Nts on the stability of cleavable complexes induced by CPT

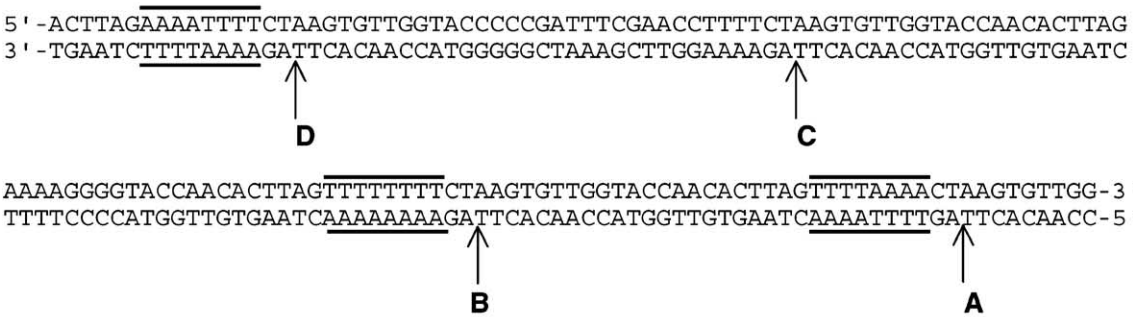
The DNA restriction fragment described above was also used to study the effects of bis-Nts on the top I-mediated DNA cleavage induced by 10 μM CPT. The presence of CPT changed the cleavage pattern by induction of four new major sites designated as E, F, G, and H (Fig. 4). Moreover, some new minor cleavage bands surrounding the sites B, C, D, F, and G were revealed. CPT-induced DNA cleavage at sites E, F, and G at comparable rates. This group of sites exhibited a high sequence homology and contained a guanine base immediately 3′ to the cleaved bond. It is noteworthy that CPT also induced a weak cleavage site H with an unique DNA sequence, which was characterized by the presence of a cytosine base at position −1.

Since the ability of each bis-Nt to inhibit the top I-mediated DNA cleavage depended on the sequence downstream of a cleavage site, we studied the site-by-site bis-Nt modulation effect on the CPT-induced DNA cleavage by top I. Bis-Nt(→→), bis-Nt(→←) and bis-Nt(←→) at a concentration of 2 or 10 μM were incubated with the substrate, and then 10 μM CPT and top I were added. Fig. 4 shows that all bis-Nts modulated (either stimulated or suppressed) the CPT-induced DNA cleavage. In the presence of CPT, bis-Nts did not inhibit the strong top I cleavage sites B, C, and D (Fig. 4). Moreover, the strong site A was found to be enhanced by CPT in the presence of bis-Nt(←→) (Figs. 4 and 5). It should be noted that the downstream region of this top I strong cleavage site contained the sequence for preferential binding of bis-Nt(←→) at position from (+3) to (+10).

The ability of bis-Nts to modulate the CPT-induced DNA cleavage by top I decreased in the following order: bis-Nt (←→) > bis-Nt (→←) > bis-Nt(→→). However, the ligands' effects on the DNA cleavage were found to be determined not only by bis-Nts molecular structures, but also by the local DNA sequences around the site of cleavage (Fig. 5). Thus, all of the three bis-Nts markedly inhibited the CPT-induced site H in a concentration-dependent manner. Conversely, all the drugs enhanced the CPT-induced sites E, F, and G, this effect inversely depending on the bis-Nts concentration (Fig. 5). In addition, bis-Nt(←→) is the most effective enhancer of this group of sites. Therefore, another key question concerns the



Panel I



Panel II

5′- TTTTAAAA – 3′ : bis-Nt(←→)
5′- AAAATTTT – 3′ : bis-Nt(→←)
5′- AAAAAAAA – 3′ : bis-Nt(→→)

Fig. 2. The effect of bis-Nts on top I-linked DNA breaks. The DNA fragment obtained by cleaving plasmid 1454 with *Xba*I and *Mlu*I. The fragment ^{33}P-labeled at its 3′-end (line 1) was incubated in the presence of purified human top I (line 3). Top I cleavages were induced in the presence of bis-Nt(→→), bis-Nt(→←), bis-Nt(←→) at a concentration of 0,2 μM (lines 4, 8, 12), 1 μM (lines 5, 9, 13), 5 μM (lines 6, 10, 14) or 25 μM (lines 7, 11, 15). Lines 2 and 16, the chemical sequence A + G. Horizontal lines indicate the location of top I cleavage sites designated by letters A–D. The sequences of bis-Nts preferential binding are underlined. Panel I schematically shows double-stranded OLIGs constituting the DNA fragment used in this experiment. Vertical arrows indicate the location of top I cleavage sites designated by letters A–D. Panel II shows the preferred binding sites on DNA for all bis-Nts studied.

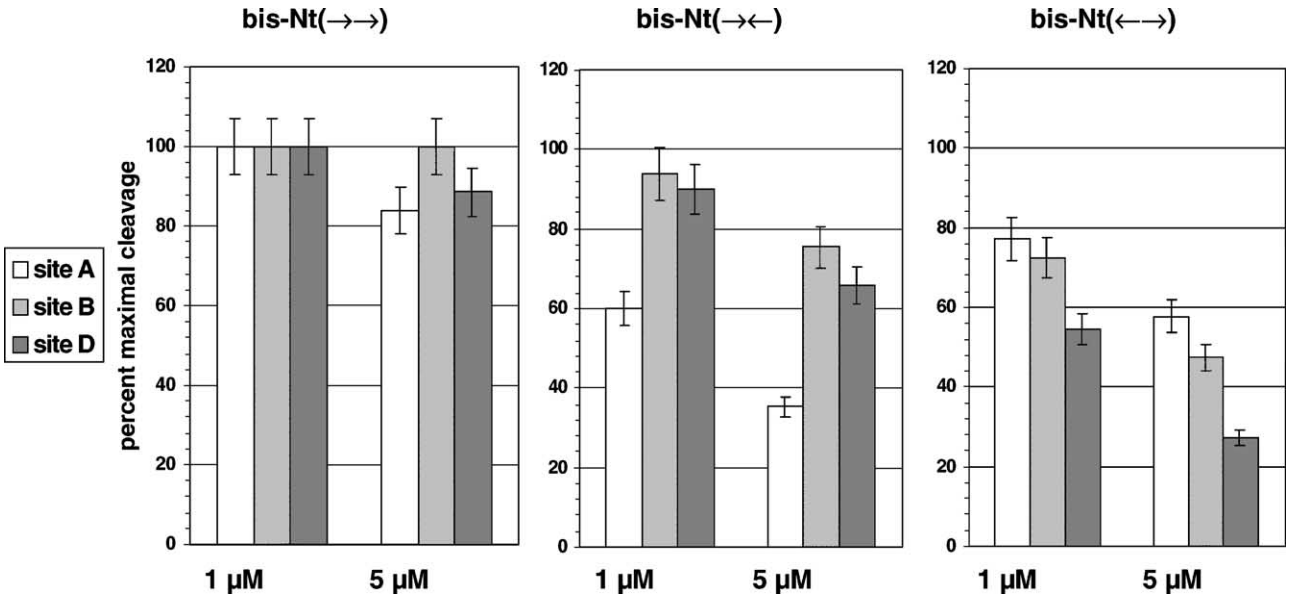


Fig. 3. Densitometric comparative analysis of the rates of DNA cleavage at the top I-specific sites A, B, and D in the presence of 1 or 5 μM of bis-Nts.

differential structural effects of bis-Nts on the DNA level, which may influence the catalytic activity of top I.

3.3. Raman spectroscopy of the OLIGs and bis-Nts complexes in solution

The spectra of OLIGs, contain the Raman bands that are characteristic of the B-form of DNA (Fig. 6). The B-form-associated Raman spectra contained a 1092 cm^{-1} band, which corresponds to the symmetric stretching vibration of the PO_2^- moiety, and two bands at 837 cm^{-1} and ca. 789 cm^{-1}, which are due to a complex vibrational mode involving the deoxyribose-linked phosphodiester network (5′COPOC3′) [26–30]. The spectra of OLIGs were almost the same at the positions of bands and were typical of DNA with the given base composition (20% G + C) [26]. The spectrum of OLIG3 exhibited a 2–3 cm^{-1} downshift of dA-sensitive vibration bands at 728 and 1376 cm^{-1} [26–28], as well as that of the 789 cm^{-1} band, as compared to the spectra of OLIG1 and OLIG2. This downshift was probably due to the long non-alternate (dA)(dT) tract of OLIG3, which was absent in the other OLIGs. Obviously, the stacking interaction and backbone parameters of this long stretch considerably differed from those for the sequences of other OLIGs. Minor differences were also observed in the relative intensities of the bands sensitive to conformation of the deoxynucleotides. This was the case, e.g. with the 670/680 and 1333/1340 cm^{-1} doublets. These doublets are of special interest being sensitive to the dG furanose ring conformation [29,30] and their perturbations reflect the long-range alteration of OLIG conformation in the sequences that different from the AT sequences within the bis-Nts loading position. These bands can be used for estimation of the quantity of C2′-endo/anti (the bands at 680 and 1333 cm^{-1}) and C1′-exo/anti (the bands at 670 and 1340 cm^{-1}) conformers of dG within the OLIG sequence [29]. Comparison of Raman spectra showed that the OLIG3 spectrum had slightly more intense bands at 680 and 1333 cm^{-1} (Table 1). These variations further confirm that the conformation of OLIG3 somewhat differs from those of OLIG1 and OLIG2, although it still corresponds to the canonical B-form.

Raman spectral analysis of the changes in the spectra of bis-Nt–OLIG complexes provides evidence for mutual adjustment of the OLIGs and bis-Nts molecular structures. The structural perturbations of the bis-Nts molecules were analyzed in detail in [31]. Briefly, these are the reorientation of the pyrrole rings and changes of the torsion angles of peptide bonds, which are strongly dependent on the bis-Nt–OLIG binding specificity. These conclusions are supported by X-ray [10] and NMR [11] data. The most considerable alterations in the spectra of the bis-Nt–OLIG complexes were associated with variations in the intensity of the bands of doublets at 680/670 and 1333/1340 cm^{-1} (Table 1). The binding of bis-Nt(←→) and bis-Nt(→←) resulted in an increase in the relative intensity of the bands at 680 and 1333 cm^{-1}. Besides, the increase in the relative intensity was more pronounced in the spectra of complexes of bis-Nt(←→) and bis-Nt(→←) with OLIGs that are non-specific for corresponding drugs. This suggests that bis-Nt(←→) and bis-Nt(→←) induced alterations in the OLIG conformation in the sequences that are different from the bis-Nts loading position and are locates in the regions remote from it. We did not find such noticeable spectral variations for the complexes of bis-Nt(→→), which is likely to be due to the relatively flexible linker of this derivative. Conversely, bis-Nt(←→) binding to DNA results in a more pronounced increase in the intensity of dG C2′-endo/anti bands compared to that for bis-Nt(→←).

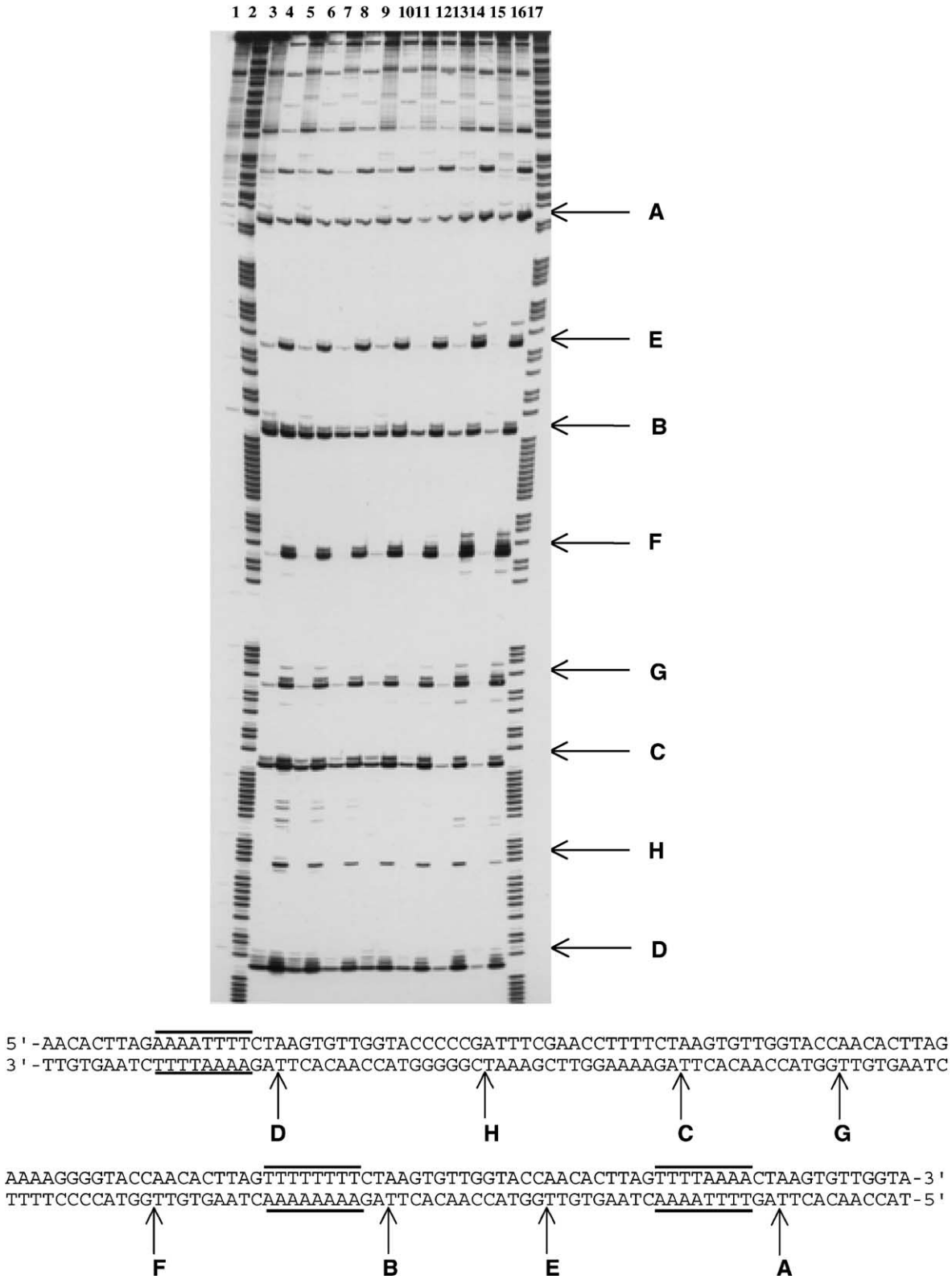


Fig. 4. Modulation of top I-mediated DNA cleavage by CPT in the presence of bis-Nts. The DNA fragment obtained by cleaving plasmid 1454 with *Xba*I and *Mlu*I. The fragment ^{33}P-labeled at its 3′-end (line 1) was incubated in the presence of purified top I and in the absence (line 3) or in the presence (line 4) of 10 μM CPT. Top I cleavage was induced in the presence of bis-Nt(→→) (lines 5, 7), bis-Nt(→←) (lines 9, 11), bis-Nt(←→) (lines 13, 15) at a concentration of 2 or 10 μM, respectively. The other lines correspond to DNA fragments treated with top I in the presence of 10 μM CPT and 2 or 10 μM bis-Nt(→→) (lines 6 and 8), bis-Nt(→←) (lines 10 and 12) or bis-Nt(←→) (lines 14 and 16). Lines 2 and 17, the chemical sequence A + G. Horizontal lines show the location of top I cleavage sites designated by letters A–H. The sequences of bis-Nts preferential binding are underlined.

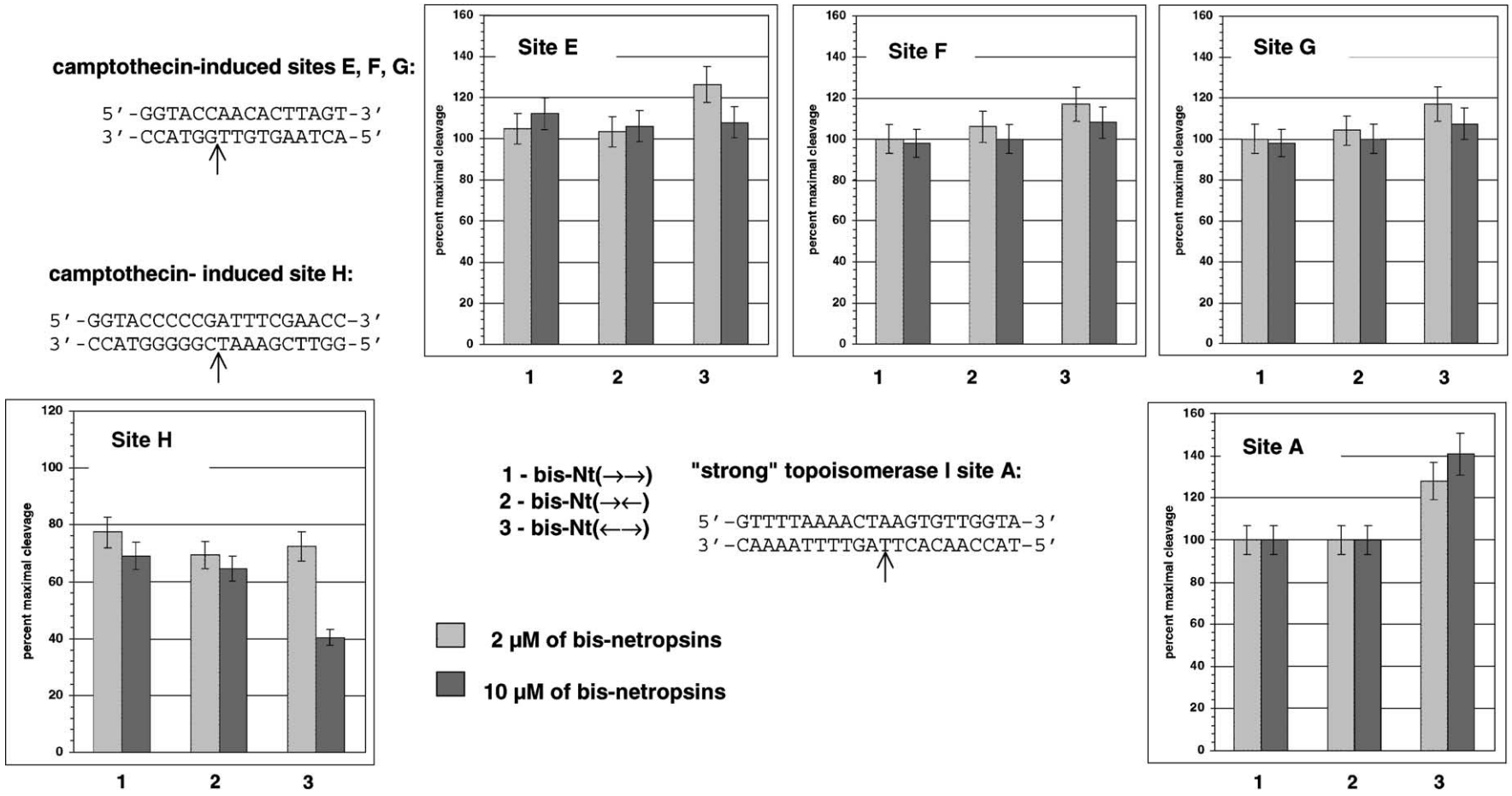


Fig. 5. Densitometric comparative analysis of the effects of bis-Nts on the top I-mediated DNA cleavage in the presence of 10 μM CPT.

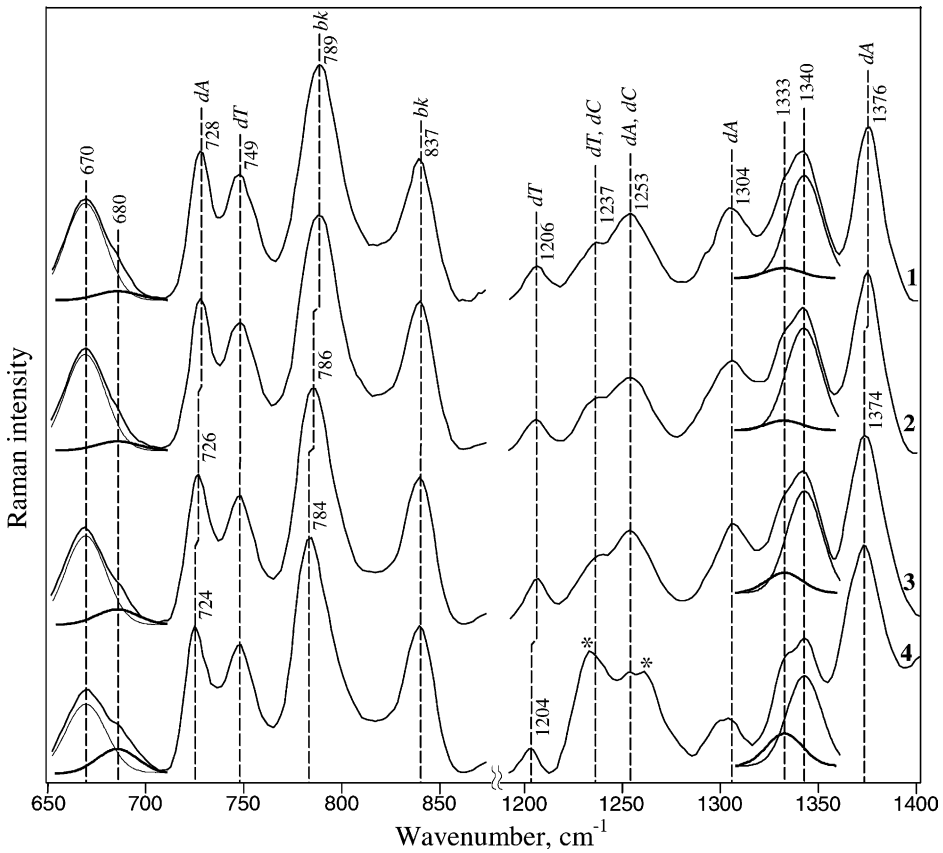


Fig. 6. Raman spectra of the OLIGs. Raman spectra of OLIG1 (1), OLIG2 (2), OLIG3 (3) and the bis-Nt(←→)–OLIG3 complex (4). The non-specific complex of bis-Nt(←→) with OLIG3 was chosen for this figure because the changes in the spectrum of OLIG after the ligand binding were the most distinct in this case. The bands of bis-Nt(←→) in the spectrum of the complex are indicated with asterisks. The spectral decomposition demonstrates the intensity perturbations of dG conformation-sensitive doublets at 680/670 and 1333/1340 cm^{-1}. The assignments of Raman bands are adapted from [26,28,30]. The labels indicate the assignment of the bands to base (C, T, A and G) or backbone (bk) residues.

Spectral analysis demonstrated the strongest ability of bis-Nt(←→) to change the OLIG structure upon binding. Specifically, the downshift of the dT conformation-sensitive band at 1206 cm^{-1} [29] was observed in all bis-Nts–OLIGs spectra and is more discernible in the spectra of bis-Nt(←→)–OLIG complexes. This downshift is also important because it reveals the conformation changes of the OLIGs within the bis-Nt-specific AT-reach sequence. Finally, the spectra of bis-Nt(←→)–OLIG complexes showed a downshift of the bands at 726 and 786 cm^{-1} only in the spectrum of bis-Nt(←→)–OLIG3, which was due to the change in the stacking interaction of adenine bases and variation of backbone parameters, respectively.

Table 1
Relative intensities of the 680/670[a] and 1333/1340 cm^{-1} bands in Raman spectra of OLIGs and bis-Nts–OLIGs complexes

	Non-bound OLIGs	Bis-Nt(←→)[b]	Bis-Nt(→←)	Bis-Nt(→→)
OLIG1				
I_{680}/I_{670}	0.09	**0.17**	0.33	0.10
I_{1333}/I_{1340}	0.09	**0.20**	0.30	0.12
OLIG2				
I_{680}/I_{670}	0.10	0.35	**0.18**	0.11
I_{1333}/I_{1340}	0.10	0.32	**0.19**	0.12
OLIG3				
I_{680}/I_{670}	0.16	0.39	0.32	**0.18**
I_{1333}/I_{1340}	0.20	0.40	0.25	**0.22**

[a] The bands at 680 and 1333 cm^{-1} arise from furanose ring C2′-endo/anti of dG, the band at 670 cm^{-1} relates to furanose rings C1′-exo/anti of dG plus C2′-endo/anti of dT, and the band at 1340 cm^{-1} is assigned to vibration of the ring C1′-exo/anti of dG with a contribution of the 1342 cm^{-1} band of adenine.

[b] The cases of bis-Nt-specific binding to OLIGs are marked in bold.

4. Discussion

New synthetic bis-Nts studied in this work differ from one another in the symmetry of orientation of their Nt moieties, rigidity of their linkers, molecular groups at the termini and an existence of Pt-atom in one of the agents (Fig. 1). Previous studies demonstrated that the only orientation of the netropsin's monomers and rigidity of the linker are responsible for the sequence-specificity of drug dimers DNA-binding whereas the other molecular groups of the ligands have the impact in the non-specific affinities [12–14,19–22]. All of the three bis-Nts were shown to be effective modulators of the top I-mediated DNA cleavage [18].

It is known that bis-Nts may bind to DNA sequences containing AT tracts of eight base pairs with the affinities determined by the polarity of the AT tract [4,32]. As was described previously, bis-Nt(←→) binds preferentially to the T_4A_4 DNA sequence and less strongly to the A_4T_4 block. However, Nt monomers of the bis-Nt(→←) molecule are oriented in the opposite direction relative to those of bis-Nt(←→), and its strongest affinity binding site should be A_4T_4. Preferred binding sites for bis-Nt(→→) are A_8 or T_8 sequences [20,21]. Footprint analysis correlating the position of bis-Nts binding to specific AT tracts with the induction of DNA cleavage by topo I has been described by us previously [20–22].

Fig. 2 shows that the symmetrical bis-Nt(←→), which has the most rigid linker, is the most effective suppressor of the top I-mediated DNA cleavage. Raman spectroscopy data demonstrate that this compound induced the strongest, among all bis-Nts, alterations of the DNA substrate structure upon binding (Table 1). Moreover, this ligand induced the strongest DNA structure alterations at its loading position in the AT-reach DNA sequence and remote DNA alterations, including variations in the C2′-endo/anti conformation of the guanine base.

NMR spectroscopy and X-ray studies on the conformation and relative degree of bending in the series of DNA duplexes and their complexes with sequence-specific proteins demonstrate that the minor groove of the oligo(dA) tract (defined as four or more successive A_n or T_n) is compressed gradually from 5′ to 3′ [33] leading to formation of an unique structure of a quintessential rigid rod [34] and its stable curvature [33]. Inclusion of the TA step into the sequence disrupts the compression of the minor groove of the tract and induces positive roll bending in B-DNA due to compression of the large and relatively open major groove. In general, this TA step is analogous to a flexible hinge: it can bend if the necessary stress is applied [34]. On the other hand, the capacity of the AT region for bending may be modulated by interaction of the minor groove binders with DNA through the ligand-induced widening of the minor groove [20] expecting to be maximum if the interaction of the ligand with DNA is characterized by the lowest mutual stereo-conformity. This explains our spectral data on the selectivity of the bis-Nts binding to their ''specific'' and ''non-specific'' DNA substrates.

Indeed, the local perturbations in the DNA structure were the greatest when the ligand and OLIG lacked mutual stereo-conformity (the interactions of bis-Nts with the corresponding ''non-specific'' OLIG). Binding of Nt moieties of bis-Nts to AT tracts is stereo-specific with respect to the C5′–C3′ direction of the polynucleotide chain [14]. This is why non-symmetrical bis-Nt(→→) induces smaller DNA alterations than bis-Nt(←→) and bis-Nt(→←). The symmetrical bis-Nt(←→) and bis-Nt(→←) induce significantly greater DNA alterations than bis-Nt(→→) does. Raman spectroscopy demonstrates that they can cause pronounced alterations in the corresponding ''non-specific'' OLIGs whereas their binding to ''specific'' OLIGs does not induce any changes in the DNA structure spectral markers (Fig. 6).

The fact that bis-Nt(←→) with a rigid linker and a centrally located Pt(II) atom induces minimal DNA structural alterations of its specific OLIG1 may be explained by the presence of a TA step in OLIG1. This TA step should cause DNA bending and widening of the minor groove, and facilitate binding of ''non-flexible'' bis-Nt(←→). On the contrary, bis-Nt(→←) contains a long and flexible linker and may be adopted by the OLIG2, which has a more rigid structure.

Fig. 3 shows a certain site selectivity of the inhibition of the top I cleavage activity by low concentrations of bis-Nts. These top I cleavage sites differ in the polarity of the AT tract downstream of top I cleavage site. We have found that all bis-Nts inhibit top I-mediated DNA cleavage more effectively at the sites that do not provide a steric arrangement for selective binding of the drug. Therefore, bis-Nts binding to non-preferential AT sequences may eliminate the enzymatic activity through either elimination of the structural features downstream of the cleavage site that are important for top I catalysis or induction of DNA conformational changes. It is known that eukaryotic top I mediates DNA cleavage at the preferred consensus sequences. DNA substrates used in this work include two separate DNA duplex regions relative to the top I cleavage site: the first one is a degenerate consensus that includes the cleavage site and determinates the basal level of DNA cleavage by top I in the absence of NaCl [35], and the other is an AT tract downstream of the cleavage site, where a specific helix curvature and positioning of the bend may modulate the cleavage efficiency [36].

The top I crystal structure [37] indirectly indicates the top I sensitivity to the DNA conformation downstream of the cleavage site. Top I in a non-covalent and covalent complexes with 22 bp OLIG forms only a few contacts with the DNA phosphate of scissile and non-cleaved strands downstream of the cleavage site, whereas two positively charged enzyme's surfaces (helices 5 and 6 of the core subdomains I and II and the coiled-coil linker domain) are facing the downstream region of the DNA substrate [37,38]. In addition, the ends of the DNA duplex in a complex with top I are shifted by as much as 5.4 Å perpendicular to the DNA helical axis, which suggests a pronounced flexibility of interaction. This fact indicates a dependence of the top I catalytic activity on the plasticity of its DNA substrate [39]. The top I sensitivity to an overall flexibility of the DNA points to the intriguing possibility that DNA bending modulates the activity of top I, being involved in a mechanism by which a number of vital processes in cells are regulated. Indeed, bis-Nts may serve as a prototype of gene-selective drugs due to its capacity for selective inhibition of top I-mediated cleavage at different DNA sites.

Numerous data indicate that the different human cancer cell lines overexpressing top I are sensitive to top I poisons

and cell killing is related to formation of cleavage complexes [40]. We have demonstrated the modulation effects of bis-Nts on the formation of ternary complexes in the presence of CPT. Three findings are illustrated by Figs. 4 and 5.

First, all bis-Nts are able to inhibit CPT-induced cleavage at the site containing the AT stretch (site H) located closely to the top I-induced DNA single break. It should be noted that the DNA fragment that we used as a top I substrate contains only 1 week CPT-dependent site, which has an artificial structure and is characterized by the presence of a cytosine base at position −1 and 3′-T_1A_3 immediately upstream of cleavage. Actually, two Nt-like fragments of bis-Nts may act independently upon binding and a monodentate binding of the ligands is also possible [32]. In this case, the drugs may bind to any sequence that contains more than three successive AT base pairs, and hence, inhibit a ternary complex formation at sites like H.

Second, none of bis-Nts inhibits the strong top I sites in the presence of CPT. Moreover, selective binding of the most active compound, bis-Nt(←→), to the AT tract downstream of the top I cleavage site enhances the ternary complex formation (site A). In the case of preincubation of bis-Nts with the DNA, this effect directly depends on the concentration of the drug, and its rate increases to the saturation level for ligands–DNA-binding. It is likely that the bis-Nts-induced alterations in the DNA AT tracts inhibit top I-mediated DNA cleavage in the absence of CPT but promote formation of ternary complexes in the presence of CPT.

The third finding is the enhancement of the CPT-induced top I-mediated DNA cleavage by bis-Nts at classical CPT-dependent sites with a guanine base immediately 3′ to the cleaved bond. This effect is evident for the low concentrations of ligands and disappears after an increase of concentration (Fig. 4). We would like to note that the DNA sequences adjacent to these cleavage sites do not contain AT stretches (the bis-Nts loading positions). Thus, participation of bis-Nts in the enhancement of the CPT-induced top I-mediated DNA cleavage should be long-distant. This long-distant effect may be accounted for by the ability of bis-Nts to stabilize the structure or to increase the DNA rigidity. The top I-DNA affinity presumably depends on the rigidity of its DNA substrate. Thus, the enhancement of the CPT-induced cleavage site may be explained by the bis-Nts-mediated stabilization of the DNA structure.

Our results suggest that the bis-Nts cause sequence-specific inhibition of top I-mediated DNA cleavage at the sites with a certain structure of AT tracts downstream of the cleavage site. We have also shown that bis-Nts are able to stabilize CPT–top I–DNA ternary complexes at specific sites. The data may be important for development of small sequence-specific DNA-binding molecules capable of binding the regulatory element of a gene and controlling the gene expression. These molecules may be also used for cancer treatment due to their ability to selectively inhibit enzymes of the cellular transcription or replication machinery. In addition, the data obtained suggest the possibility of using bis-Nts with desired functions as pretreatment agents in cancer chemotherapy for increasing the selectivity and effectiveness of top I poisons.

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