ISSN 0006-2979, Biochemistry (Moscow), 2010, Vol. 75, No. 6, pp. 695-701. © Pleiades Publishing, Ltd., 2010.
Original Russian Text © O. Yu. Susova, A. A. Ivanov, S. S. Morales Ruiz, E. A. Lesovaya, A. V. Gromyko, S. A. Streltsov, A. L. Zhuze, 2010, published in Biokhimiya, 2010, Vol. 75, No. 6, pp. 781-788.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM09-347, May 9, 2010.

Minor Groove Dimeric Bisbenzimidazoles Inhibit *in vitro* DNA Binding to Eukaryotic DNA Topoisomerase I

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Received November 30, 2009
Revision received December 14, 2009

Abstract—The potential of six dimeric bisbenzimidazoles bound to scDNA to inhibit eukaryotic DNA topoisomerase (topo-I) was studied chemically; the tested compounds differed in linker structure and length. All the compounds inhibited topo-I, DB(7) being the most efficient; its inhibitory activity *in vitro* was 50-fold higher than that of camptothecin. It is noteworthy that inhibitory properties of nearly all the tested compounds increased many times if they were preincubated with scDNA for three days.

DOI: 10.1134/S0006297910060039

Key words: dimeric bisbenzimidazoles, topoisomerase I, inhibition

DNA topoisomerase I (topo-I) is one of the key enzymes responsible for cell vital activity. Topo-I governs double-stranded DNA (dsDNA) topology during transcription, replication, recombination and repair processes. The enzyme relaxes supercoiled DNA (scDNA) by insertion of nicks with subsequent restoration of covalent integrity by ligation of the scDNA [1]. The alkaloid camptothecin is a conventional topo-I inhibitor [2, 3]. Its synthetic analogs topotecan (Hycamtin®) and irinotecan (Camptosar®) are used in medical practice as antitumor drugs [4].

Now topo-I is an accepted molecular target for many compounds with antitumor activity [5, 6]. In recent years several novel groups of compounds—indolocarbazoles, protoberberines, indenoisoquinolines, and other topo-I inhibitors—have been actively studied and clinically tested. Their mechanism of action is based on intercalation between DNA base pairs [7]. In fact, such interaction of indolocarbazoles and indenoisoquinolines with DNA is a breakpoint of its sugar-phosphate backbone, and their ability for binding both to DNA and enzyme was shown by X-ray structural analysis of DNA–topo-I complex [8].

To obtain novel efficient antitumor and antiviral preparations, much attention is now given to synthesis and study of compounds capable of noncovalent and site-specific binding to dsDNA. Low molecular weight compounds interacting with dsDNA via the minor groove are the most promising for this purpose. These compounds are substantially free of mutagenic side effect [9, 10] typical of low molecular weight compounds intercalating between dsDNA base pairs [11]. The antibiotic netropsin and distamycin A and fluorescent dyes Hoechst 33258 and Hoechst 33342 are the most intensively studied minor-groove compounds [12, 13].

Successful elucidation of molecularly based AT specificity of these antibiotics [14, 15] and Hoechst 33258 dye [16, 17] binding to dsDNA opened the way to construction and directed synthesis of new types of ligands with high affinity and specificity to dsDNA [18-21]. Some minor groove ligands are now clinically tested as antitumor, antiviral, and antibacterial drugs [22]. Earlier we showed that dimeric analogs of the antibiotic netropsin—bis-netropsins with various chemical compositions synthesized on its basis—appeared to be efficient modulators of eukaryotic topo-I activity against dsDNA

Abbreviations: dsDNA, double-stranded DNA; scDNA, supercoiled DNA; topo-I, DNA topoisomerase I.

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[23, 24]. This work is the first *in vitro* study of topo-I minor groove inhibitors belonging to a new group of compounds—dimeric bisbenzimidazoles. Our goal was to answer two questions: (i) whether dimeric bisbenzimidazoles inhibit topo-I more actively than their monomers; (ii) what types of scDNA complexes with dimeric bisbenzimidazoles are most active as topo-I inhibitors.

That is why this work precedes further studies of these molecules on the cell level and is also necessary for directed synthesis of more active new compounds of this class.

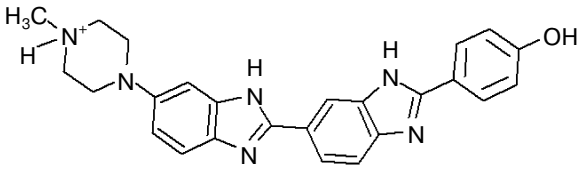
In this work we studied the following compounds as topo-I inhibitors: symmetrical dimeric bisbenzimidazoles **DB(*n*)** including their derivative **bis-HT(NMe)** and monomeric bisbenzimidazole **MB**. Chemical structures of **MB**, **bis-HT(NMe)**, and **DB(*n*)** are given below.

The synthetic procedure will be published as a separate paper. **Bis-HT(NMe)** consists of two Hoechst 33258 molecules linked by the flexible linker -$(CH_2)_3$-N(Me)-$(CH_2)_3$-. **DB(*n*)** consists of two **MB** molecules linked by oligomethylene linkers of various length (n = 3, 4, 5, 7, 11) (see Scheme).

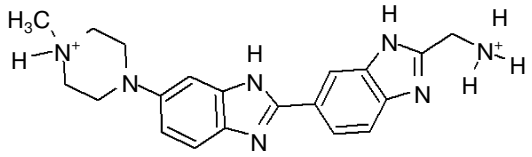
The synthesized dimeric bisbenzimidazoles appeared to be efficient topo-I inhibitors *in vitro*. It is shown that dimeric bisbenzimidazole **DB(7)** is tens of times more efficient eukaryotic topo-I inhibitor than camptothecin. Inhibitory activity of dimeric bisbenzimidazoles was found to increase significantly if they were preincubated with scDNA.

MATERIALS AND METHODS

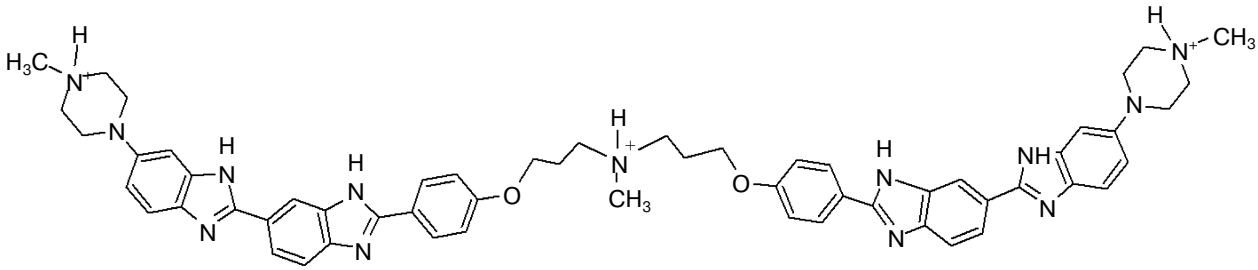
Preincubation of compounds. The following procedure was used to obtain mainly open linear and/or sandwich complexes of dimeric bisbenzimidazoles with scDNA. Solutions of dimeric bisbenzimidazoles were prepared in DMSO at ~4-5 mM concentrations. Since buffer solutions of dimeric bisbenzimidazoles were used in experiments, they were stored for three days at room temperature for better dissolution of the compound (at double the required concentration). Then equal volumes of dimeric bisbenzimidazole and plasmid scDNA solutions were mixed and stored for three days at 4°C to obtain thermodynamic equilibrium mixtures of complexes with scDNA. Before insertion of the enzyme, the mixture was heated for 15 min at 37°C. The third (aggregated) type of dimeric bisbenzimidazole–scDNA complexes was obtained by dilution of compounds to the required concentrations directly before the reaction; the latter was



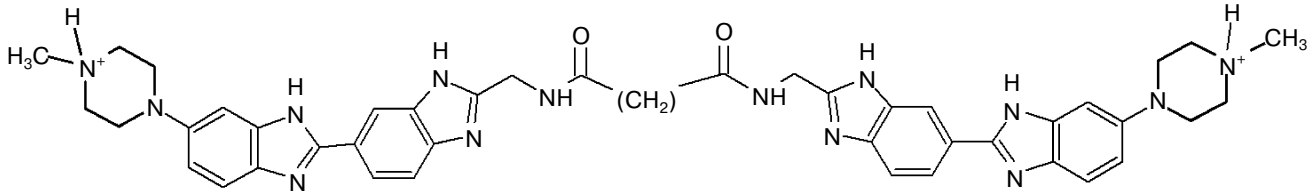
Hoechst 33258



MB



bis-HT(NMe)



DB(3), n = 3; DB(4), n = 4; DB(5), n = 5; DB(7), n = 7; DB(11), n = 11

initiated by simultaneous addition of topo-I to all samples.

Inhibition of catalytic activity of eukaryotic topoisomerase I in the relaxation reaction of supercoiled DNA. Modulation of topo-I activity *in vitro* was studied using the Topoisomerase I Drug Screening kit (TopoGen). Two units of purified topoisomerase from calf thymus (Fermentas, Lithuania) and the studied compounds were incubated with 0.15 μg of supercoiled plasmid DNA pHOT1 (TopoGen) in reaction buffer (10 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.15 M NaCl, 0.1% BSA, 0.1 mM Spermidine, 5% glycerol). The mixture was incubated for 30 min at 37°C, the reaction was stopped by addition of SDS to the final concentration 1%, and the reaction mixture was treated by proteinase K at the final concentration 50 μg/ml for 30-60 min at 37°C. The reaction products were separated electrophoretically in 1% agarose gel with TAE buffer (2 M Tris-base, 0.05 M EDTA, 1.56 M acetic acid) at the maximal voltage 3-4 V/cm. The gel was then stained with 0.1 μg/ml ethidium bromide. The presence of DNA in the gel was visualized by UV fluorescence with wavelengths from 240 to 360 nm. In the absence of inhibitor, topo-I relaxed scDNA with formation of a series of relaxed topoisomers. Topo-I inhibition was revealed by the ability of the studied compounds to retard scDNA relaxation, that is via the decreased composition of migrating topoisomers and restoration of scDNA.

RESULTS

In this work we have studied a novel class of compounds, symmetrical dimeric bisbenzimidazoles, as topo-I inhibitors. Bisbenzimidazoles and their derivative Hoechst 33258 are relatively hydrophobic compounds poorly soluble in water. The compounds were preincubated to increase solubility: dimeric bisbenzimidazoles were diluted to micromolar concentrations and stored for three days at room temperature. This time period was enough for establishment of thermodynamic equilibrium of various aggregate forms of the dimeric bisbenzimidazoles in solution. Then the studied compounds were incubated with plasmid scDNA for the next three days for re-establishment of thermodynamic equilibrium in the presence of DNA.

Initially we compared *in vitro* inhibitory activity against topo-I of Hoechst 33258 and its new dimeric analog **bis-HT(NMe)** without their preincubation with scDNA (Fig. 1). In the absence of inhibitor insertion of the purified topo-I results in complete relaxation of scDNA with formation of a set of topoisomers differing by engagement order (Fig. 1, lane *2*). At concentrations from 2.5 to 10 μM, Hoechst 33258 insignificantly inhibited topo-I with a negligible compositional deviation of relaxed topoisomers. As for **bis-HT(NMe)**, at concentration 10 μM it almost completely inhibited scDNA relaxation by topo-I, retaining most of the DNA in supercoiled form. Thus, it was found that one of the dimeric bisbenzimidazoles possesses significantly higher activity than its monomeric form. This supports our strategy for synthesis of dimeric bisbenzimidazoles as highly active topo-I inhibitors.

Then we studied the influence of preincubation of the tested dimeric bisbenzimidazoles with scDNA on their ability to inhibit topo-I activity. Preincubation of **bis-HT(NMe)** with scDNA significantly enhanced topo-I inhibition compared with the experiment without preincubation (Fig. 2a). Enhanced topo-I inhibition was also observed after preincubation of three dimeric bisbenzimidazoles, **DB(3, 4, 11)**, with scDNA (Fig. 2b). For **DB(3)** and **DB(4)**, the effect was clearest. In fact, at their concentration 2.5 μM in the presence of topo-I relaxation of DNA retarded and most of the DNA remained in the

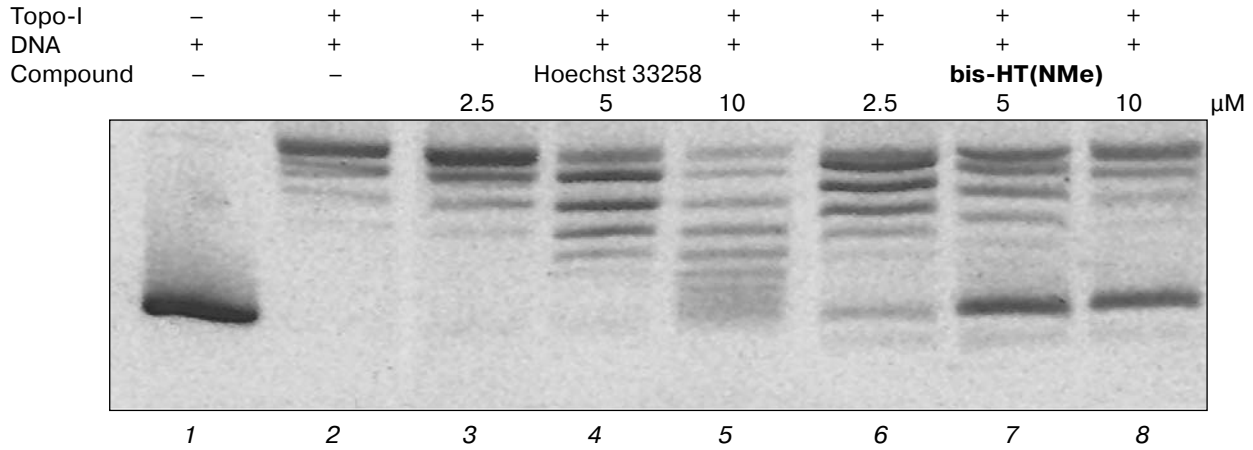


Fig. 1. Comparison of topo-inhibitory activity of Hoechst 33258 and its new analog **bis-HT(NMe)**. Topo-I DNA relaxed in the presence of Hoechst 33258 and **bis-HT(NMe)** for 30 min at 37°C. Addition of topo-I into the reaction mixture initiated the reaction. Experiments were repeated at least three times.

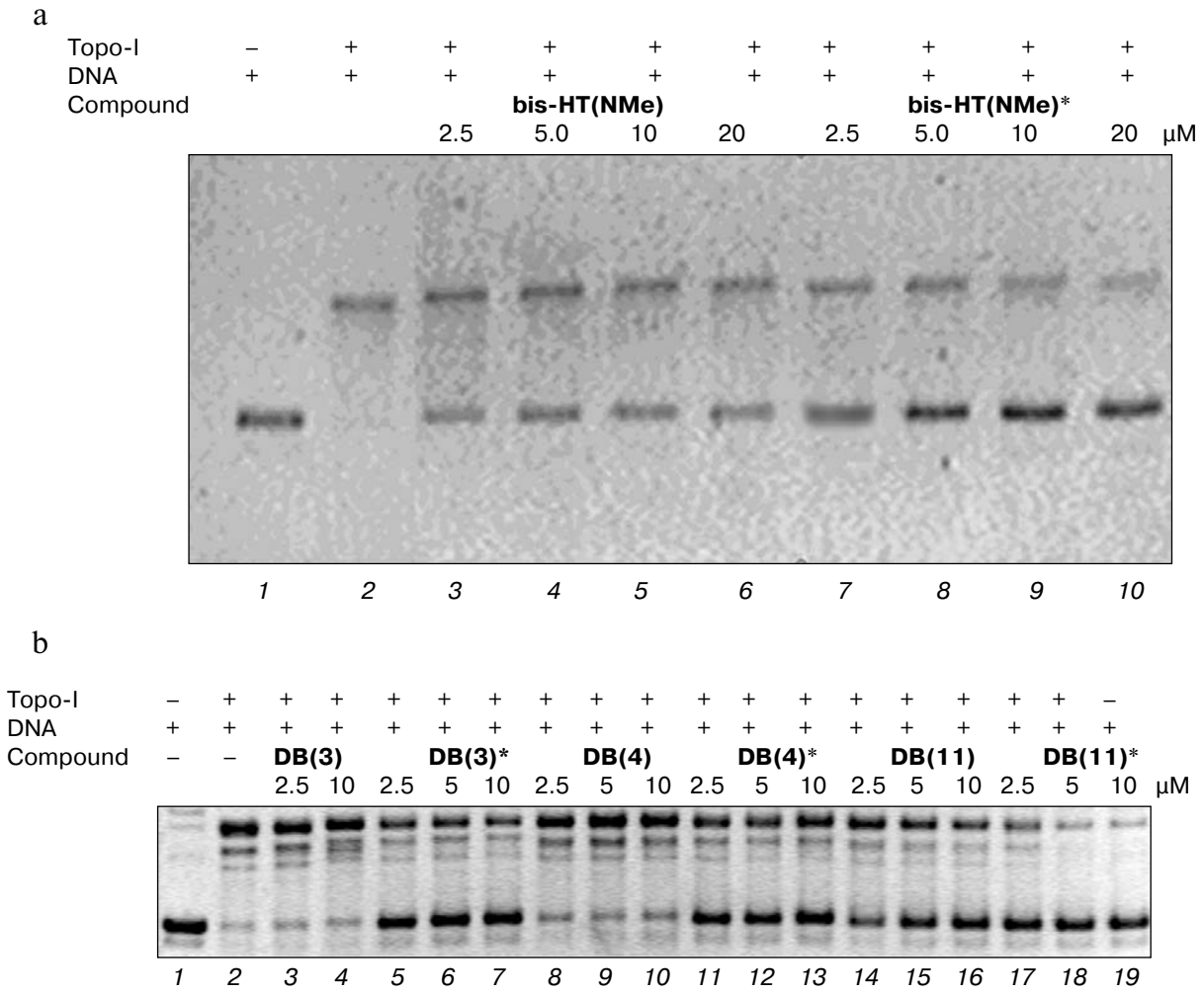


Fig. 2. Influence of preincubation of novel dimeric bisbenzimidazoles with scDNA on their efficiency as topo-I inhibitors: a) **bis-HT(NMe)** at concentrations 2.5, 5, 10, and 20 μM; b) **DB(3)**, **DB(4)**, and **DB(11)** at concentrations 2.5, 5, and 10 μM. * Compounds were stored for three days in aqueous solution at room temperature, then with scDNA for the same time at 4°C. For control experiments, the compounds were added to the reaction mixture directly before the reaction, which was initiated by addition of topo-I to the reaction mixture. Experiments were repeated not less than three times.

supercoiled form, whereas without preincubation of **DB(3)** or **DB(4)** only trace amounts of scDNA were observed even at their concentration 10 μM. Experiments with **DB(5)** gave analogous results (data not presented here). **MB** did not inhibit topo-I *in vitro* (data not presented here). It is noteworthy that **MB** appeared to be a significantly less efficient topo-I inhibitor than Hoechst 33258. The ability of **DB(11)** for topo-I inhibition was almost independent of preincubation with scDNA. The reason for this phenomenon will be discussed in the next section.

For **bis-HT(NMe)**, **DB(3)**, and **DB(4)**, the effect of preincubation on topo-I inhibition manifested itself even at concentrations 2.5 μM. That is why we studied the possible inhibitory effect on topo-I at nanomolar concentrations of dimeric bisbenzimidazoles. Using **DB(7)** as an example, we demonstrated that even at concentrations 250 nM dimeric bisbenzimidazoles are able to inhibit topo-I (Fig. 3). In spite of the fact that **DB(7)** was not the most efficient topo-I inhibitor, we demonstrated the regions of predominant binding of this ligand to scDNA in the first and/or second as well as the third type of complexes in the same figure. It is noteworthy that at **DB(7)** concentrations higher than 2.5 μM the inhibitory effect of **DB(7)** on topo-I activity decreases. This unusual phenomenon will be discussed in the next section.

We also compared the inhibitory properties of dimeric bisbenzimidazole **DB(7)** and camptothecin, a conventional topo-I inhibitor. The data are presented in Fig. 4. On interaction of **DB(7)** with scDNA, the inhibitory activity was tenfold higher than that of camptothecin. In fact, even at the presence of 0.5 μM **DB(7)** preincubated with scDNA, activity of topo-I was suppressed to the same extent as by addition of 25 μM camptothecin, accounting for the amount of scDNA unaffected by topo-I.

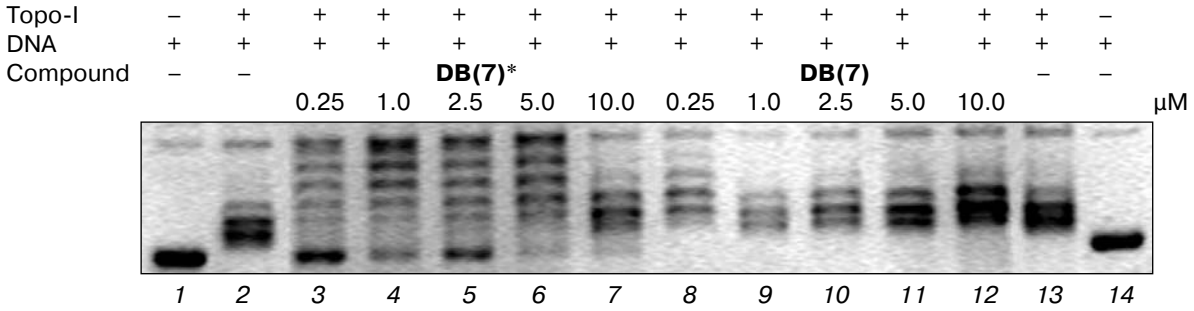


Fig. 3. Increased topo-I inhibitory effect of **DB(7)** after its preincubation with scDNA. Experiments were repeated not less than three times. The other conditions were the same as in Fig. 2.

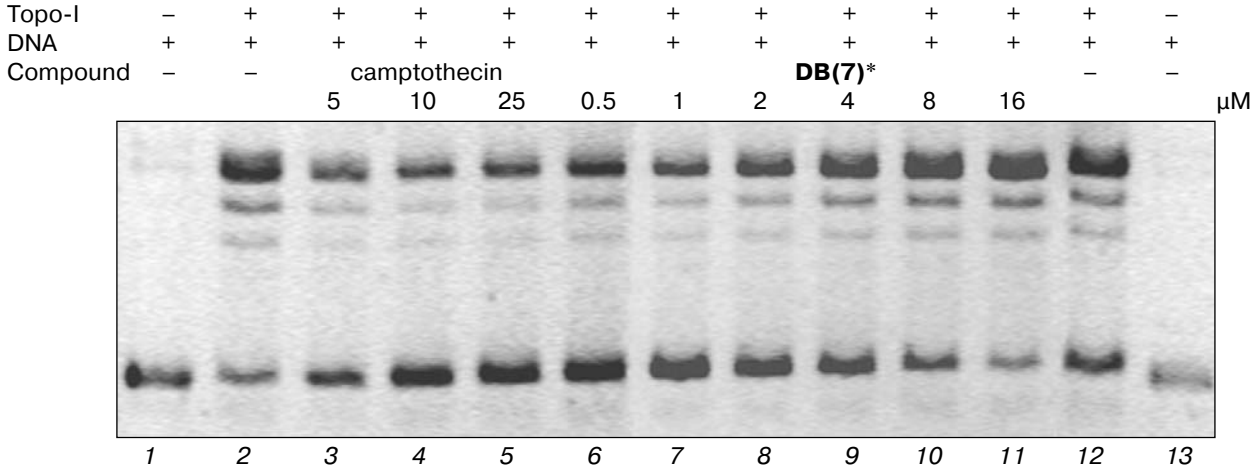


Fig. 4. Comparison of inhibitory effect on topo-I of camptothecin and **DB(7)** preincubated with scDNA. Experiments were repeated not less than three times. Other conditions were the same as in Fig. 2.

So, the novel dimeric bisbenzimidazoles efficiently inhibit *in vitro* topo-I activity. Preincubation of the dimeric bisbenzimidazoles with scDNA usually significantly increases the inhibitory activity of these compounds against topo-I.

DISCUSSION

Most of the experimental data obtained in this work can be rationalized by accounting for the results of physicochemical studies of interaction between one of the dimeric bisbenzimidazoles, **bis-HT(NMe)**, and scDNA from calf thymus. These results indicate that when **bis-HT(NMe)** concentration was increased, it formed three types of complexes on the polymer. The stoichiometry of these complexes with DNA was recognized: the first is linear opened and occupies ~11 bp on DNA; the second is an intramolecular sandwich and occupies ~5-6 bp on DNA; the third is a dimer of intramolecular sandwiches and occupies ~2-3 bp on DNA [25]. It should be noted that formation of three analogous types of DNA–Hoechst 33258 complexes was demonstrated in [26]. Earlier it was found that topotecan can form dimers in solutions and several types of complexes with scDNA. The thermodynamic equilibrium between various types of scDNA–topotecan complexes appeared to be attained after a long period of incubation. This property of topotecan also required preincubation with scDNA while studying its interaction with scDNA [27].

In our experiments, after preincubation and when scDNA was essentially filled with dimeric bisbenzimidazole, **DB(7)**, a complex of the third type was mainly formed, and the inhibitory activity of **DB(7)** against topo-I began to decrease (Fig. 3, lanes *3-7*). This indicates that complex of the third type inhibits enzymatic activity of topo-I less than complexes of the opened and/or sandwich type.

The fact that inhibitory effect manifests itself only after preincubation of dimeric bisbenzimidazole with scDNA means that transfer of **bis-HT(NMe)**–scDNA complex of the third type into more thermodynamically

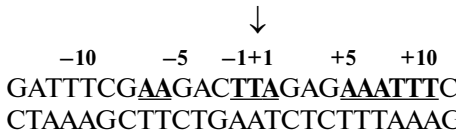
favorable complexes of the first and second types is hindered for some reason (Fig. 2a). Actually, the thermodynamic equilibrium in aqueous solution was attained after ~3 days. As a result, excess **bis-HT(NMe)** complex of the third type formed immediately after addition of the ligand in solution undergoes transformation into the opened and/or sandwich complexes [25]. This indicates that preincubation of dimeric bisbenzimidazoles with scDNA enhances their inhibitory action on topo-I.

However, the effect of preincubation on topo-I inhibition is demonstrated not only for **bis-HT(NMe)**. Topo-I was also efficiently inhibited by dimeric bisbenzimidazoles **DB(3)** and **DB(4)** preincubated with scDNA (Fig. 2b). This is not surprising because **bis-HT(NMe)** and **DB(*n*)** have similar structures.

It is noteworthy that preincubation of **DB(11)** with scDNA did not significantly influence topo-I inhibition (Fig. 2b, lanes *17-19*). Elongation of the linker to 11 methylene units in **DB(11)** possibly hinders formation of **DB(11)**–scDNA complex of the third type.

The action of monomeric bisbenzimidazole Hoechst 33258 was qualitatively different from that of **bis-HT(NMe)**. Addition of 2.5-10 μM Hoechst 33258 to scDNA caused negligible inhibition of topo-I with formation of a small number of topoisomers with the complete absence of the initial scDNA. This agrees well with the earlier data [28]: Hoechst 33258 binding to scDNA in the presence of topo-I results in the prevailing relaxed DNA form in solution, whereas addition of **bis-HT(NMe)** promoted conservation of scDNA in solution.

Plasmid pHOT1 (Topogen) used in this work contains one highly efficient cleavage site of topo-I:



This fragment represents the plasmid sequence, the cleavage point is marked by the arrow, AT sites are at the cleavage point, and the neighboring sites are underlined.

We suppose that the relatively high inhibitory activity of dimeric bisbenzimidazoles is connected with the fact that on the cleavage site of pHOT1 plasmid by topo-I is placed from (–2) to (+1) and from (+5) to (+10), and also sites from (–6) to (–7) are occupied by at least two A·T pairs. Consequently, there are two pairs of sites, and to each of these sites a half of the monomeric bisbenzimidazole molecule (**MB**) can be bound. In fact, according to the X-ray structural data, a molecule of monomeric bisbenzimidazole, Hoechst 33258, forms hydrogen bonds with two neighboring A·T pairs [29]. Thus, on two pairs of sites divided by a short sequence of G·C pairs simultaneous binding of the whole dimeric bisbenzimidazole molecule in this linear form can occur. Since our ligands bind to scDNA alongside each other or directly at the cleavage point of scDNA by topo-I, it can be suggested that these ligands are competitive inhibitors of topo-I. However, on the duplex presented above there are sites for binding halves of molecules of dimeric bisbenzimidazoles more distant from the cleavage point. Binding of dimeric bisbenzimidazoles on the sites distant from the cleavage point might change the DNA structure not only at the ligand binding point but also at some distance from it, allosterically hindering topo-I binding. That is why now it is unclear whether our compounds are competitive and/or allosteric highly efficient inhibitors of topo-I hindering enzyme placement on DNA. There are some other compounds blocking formation of topo-I–scDNA complex [30]. The absence of accumulation of nicks causing mutagenicity and gene toxicity [7] is an advantage of the tested compounds. The abovementioned properties are typical of action of camptothecin and other topo-I inhibitors called "poisons" [31].

The sandwich form of dimeric bisbenzimidazoles requires a block of 5-6 bp for its binding to scDNA. On the fragment of pHOT1 plasmid given above, there is only one such AT block at a rather long distance from the cleavage point of scDNA by topo-I. That is why participation of this form of complex in topo-I inhibition seems to be less probable. The fact that complex of the third type at concentration 2.5 μM prevails in solution supports its presence also at lower concentrations. Formation of complex of the third type decreases concentration of the active forms of dimeric bisbenzimidazoles, opened and/or sandwich, in solution. That is why we suppose that synthesis of dimeric bisbenzimidazoles possessing high biological activity should be aimed at construction of compounds with minimal ability for forming the third type of complex with dsDNA.

So, we suggest a novel class of topo-I inhibitors at least tenfold more efficient than the conventional topo-I inhibitor camptothecin. Preincubation of the dimeric bisbenzimidazoles with scDNA usually significantly increases the inhibitory activity of the compounds against topo-I. It is noteworthy that inhibitory properties of nearly all the tested compounds increased many times if they were preincubated with scDNA for three days.

This work was financially supported by the Russian Foundation for Basic Research (grants 09-04-01126-a and 07-03-00492-a), UPI (grant 08-0312148), and Molecular and Cell Biology Program of Presidium of the Russian Academy of Sciences.

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