Published in final edited form as:

J Mol Biol. 2007 January 19; 365(3): 561–569.

Topoisomerase I-Mediated DNA Cleavage Induced by the Minor Groove-Directed Binding of Bibenzimidazoles to a Distal Site

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Summary

Many agents (e.g., camptothecins, indolocarbazoles, indenoisoquinolines, and dibenzonaphthyridines) stimulate topoisomerase I-mediated DNA cleavage (a behavior termed topoisomerase I poisoning) by interacting with both the DNA and the enzyme at the site of cleavage (typically by intercalation between the –1 and +1 base pairs). The bibenzimidazoles, which include Hoechst 33258 and 33342, are a family of DNA minor groove-directed agents that also stimulate topoisomerase I-mediated DNA cleavage. However, the molecular mechanism by which these ligands poison TOP1 is poorly understood. Toward this goal, we have used a combination of mutational, footprinting, and DNA binding affinity analyses to define the DNA binding site for Hoechst 33258 and a related derivative that results in optimal induction of TOP1-mediated DNA cleavage. We show that this DNA binding site is located downstream from the site of DNA cleavage, encompassing the base pairs from position +4 to +8. The distal nature of this binding site relative to the site of DNA cleavage suggests that minor groove-directed agents like the bibenzimidazoles poison TOP1 via a mechanism distinct from compounds like the camptothecins, which interact at the site of cleavage.

Keywords

Hoechst 33258; topoisomerase I poisoning; DNase I footprinting; fluorescence-detected DNA binding; DNA bending

Human DNA topoisomerase I (TOP1) is an essential enzyme that functions as a swivel during DNA replication and transcription, as well as in chromosome segregation and condensation.^{1–5} It catalyzes the relaxation of DNA supercoils via a mechanism that involves both a cleavage and a religation reaction.⁴ A key intermediate in the catalytic cycle of the enzyme is a transient covalent enzyme-DNA complex (termed the cleavable complex) in which the active site tyrosine residue at position 723 is covalently linked to the 3'-phosphate terminus of the transiently cleaved DNA strand.⁴ Anticancer agents that target TOP1 (which we will hitherto denote as TOP1 poisons) achieve their cytotoxic effects by stabilizing and trapping the cleavable TOP1-DNA complex, which ultimately leads to the accumulation of DNA strand breaks and cell death.^{6–10}

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To date, only two TOP1 poisoning anticancer drugs are in clinical use, with both of these drugs (topotecan and irinotecan) being derivatives of the natural product camptothecin (CPT). The antitumor activities of CPT derivatives in humans are compromised by several factors including pH-dependent chemical instability^{11–14} and high affinity for human serum albumin.^{15,16} In recent years, a number of new classes of TOP1-poisoning agents have been identified, with many of these agents (including the indolocarbazoles,^{17–21} the indenoisoquinolines,^{21–24} the protoberberines,^{25–29} nitidine,^{30,31} and the dibenzonaphthyridines^{32–35}) acting by intercalation into DNA. Recent crystallographic studies have indicated that indolocarbazole and indenoisoquinoline derivatives poison TOP1 by inserting between the –1 and +1 base pairs relative to the site of cleavage and engaging in interactions with both the DNA and the enzyme.^{21–24} CPT and its derivatives exhibit a similar mode of interaction,^{21,36} which, in turn, has been shown to stimulate TOP1-mediated DNA cleavage by inhibiting the religation step of the enzyme catalytic cycle.³⁷

Among all the recently identified TOP1-poisoning agents, only the bibenzimidazoles^{38–46} (which include Hoechst 33258 and 33342) and terbenzimidazoles^{47–51} bind noncovalently in the minor groove of duplex DNA, with a preference for tracts of A•T base pairs. However, little is known about the mechanism by which these DNA minor groove-directed agents poison TOP1. In this connection, other prototypical DNA minor groove-binding agents, such as netropsin, distamycin, berenil, and DAPI, exhibit little or no TOP1 poisoning activity.⁴³ Thus, occupancy of the minor groove alone does not appear sufficient to impart TOP1 poisoning activity. Our previous studies have suggested that terbenzimidazole-induced DNA bending may be an important determinant of the TOP1 poisoning activity associated with this class of compounds.^{51,52} However, the precise DNA site of bibenzimidazole or terbenzimidazole binding that results in the stimulation TOP1-mediated DNA cleavage has never been identified. In this study, we demonstrate that the DNA binding site for the bibenzimidazole derivatives Hoechst 33258 (H33258) and 5-phenyl-2'-(indolo-6-yl)bibenzimidazole (5P2'IBB) (see structures in Figure 1) that yields optimal stimulation of TOP1-mediated DNA cleavage is located downstream from the site of cleavage and encompasses five A•T base pairs at positions +4 to +8. The distal nature of this ligand binding site relative to the site of DNA cleavage suggests that DNA minor groove binding agents like the bibenzimidazoles and terbenzimidazoles poison TOP1 via a mechanism distinct from that noted above for ligands like the CPT and indolocarbazole families of compounds, which interact at the site of cleavage.

Stimulation of TOP1-mediated DNA cleavage by H33258 and 5P2'IBB is affected by the position of an A₆•T₆ sequence relative to the site of cleavage

We designed a series of three double-stranded DNA oligomers (MG2, MG3, and MG4) containing an A₆•T₆ sequence downstream from the site of DNA cleavage (see Figure 2(a)). The 5'-end of the A₆•T₆ tract (highlighted in red in Figure 2A) occurs at a different position in each oligomer, with this site being at the +2, +3, and +4 positions relative to the site of cleavage in the MG2, MG3, and MG4 oligomers, respectively. We compared the extents to which identical concentrations (1 μM) of H33258 and 5P2'IBB stimulate human TOP1-mediated cleavage of MG2 – MG4. The resulting cleavage profiles are shown in Figure 2(b). Both ligands stimulate cleavage at the same site (denoted by an arrow) in all three oligomers, with 5P2'IBB stimulating cleavage to a greater extent than H33258. In addition, the extent to which both ligands stimulate cleavage of MG3 is greater than the corresponding extents to which they stimulate cleavage of the other two oligomers, which are similar in magnitude.

We sought to verify that the stimulation of TOP1-mediated cleavage of the MG2 – MG4 duplexes by H33258 and 5P2'IBB required the presence of the A₆•T₆ tract. To this end, we disrupted the A₆•T₆ tract of each duplex by substituting the central four A•T pairs with either G•C or C•G pairs. The sequence of the MG3 duplex in which the A₆•T₆ tract is disrupted

(MG3-DisA•T) is shown in Figure 2(c). We then determined the impact of these disruptions on the stimulation of TOP1-mediated cleavage by both H33258 and 5P2'IBB. Figure 2(d) shows representative cleavage profiles to emerge from these studies with MG3-DisA•T as the host duplex. Note that disruption of the A₆•T₆ tract abolishes the abilities of both H33258 and 5P2'IBB to stimulate TOP1-mediated cleavage. Similar results were obtained using MG2 and MG4 as the host duplexes (not shown). These observations suggest that the stimulation of TOP1-mediated DNA cleavage by both H33258 and 5P2'IBB involves interactions between the ligands and the A₆•T₆ tract located downstream from the site of cleavage. The DNase I footprinting studies described in a later section confirm these interactions.

The differing position of the A₆•T₆ tract in the MG2 – MG4 duplexes has little or no impact on the DNA binding affinities of H33258 and 5P2'IBB

We sought to compare the affinities of H33258 and 5P2'IBB for the MG2 – MG4 duplexes. To this end, we monitored the fluorescence emission intensity (*I*) of each ligand as function of added DNA. The resulting fluorescence titrations of H33258 and 5P2'IBB with each of the three DNA duplexes at 37 °C are shown in Figures 3(a) and (b), respectively. Note that binding to each DNA duplex increases the fluorescence intensity of H33258 to a similar extent, with the same being true of 5P2'IBB. We determined the DNA association constants (*K_a*) of H33258 and 5P2'IBB by analyzing the binding-induced changes in ligand fluorescence with the following formalism that is predicated on a binding stoichiometry of one ligand molecule per duplex:

$$I = I_0 + \frac{(I_\infty - I_0)}{2} \left[\left([D]_{\text{tot}} + [L]_{\text{tot}} + \frac{1}{K_a} \right) - \sqrt{\left([D]_{\text{tot}} + [L]_{\text{tot}} + \frac{1}{K_a} \right)^2 - 4[D]_{\text{tot}}[L]_{\text{tot}}} \right] \quad (1)$$

In this relationship, *I*₀ and *I* are the fluorescence emission intensities of the ligand in the absence and presence of DNA, respectively; *I*_∞ is the fluorescence emission intensity of the ligand in the presence of an infinite DNA concentration; and [D]_{tot} and [L]_{tot} are the total concentrations of DNA duplex and ligand, respectively. Equation (1) yields excellent fits of the experimental titration data (depicted as solid lines in Figure 3), with the associated correlation constants (*R*) being >0.996 in all cases. This goodness-of-fit is consistent with both H33258 and 5P2'IBB binding to each host oligomeric duplex with a stoichiometry of one ligand molecule per duplex.

The *K_a* values derived from the fits of the titration data in Figure 3 with equation (1) are summarized in Table 1. Inspection of these data reveals the following two significant features: (i) 5P2'IBB binds to each of the three host duplexes with an approximately 10-fold greater affinity than H33258. Recall that of 5P2'IBB stimulates TOP1-mediated DNA cleavage to a greater extent than H33258 (Figure 2(b)). It is likely that this enhanced TOP1 poisoning efficacy reflects the correspondingly enhanced DNA binding affinity of 5P2'IBB relative to H33258. (ii) H33258 exhibits a similar affinity for each of the host duplexes, with any differences in *K_a* being within the experimental uncertainty. The same is also true of the 5P2'IBB-DNA interactions. Thus, the differing position of the A₆•T₆ tract in the three host duplexes does not alter the binding affinity of either H33258 or 5P2'IBB.

Both H33258 and 5P2'IBB bind to an identical A₅•T₅ sequence in the MG2 – MG4 duplexes, with the location of this binding site relative to the site of TOP1-mediated cleavage being different in each of the host duplexes

The DNA binding studies described in the previous section provide important information with regard to the affinity and stoichiometry with which H33258 and 5P2'IBB bind to the MG2 –

MG4 duplexes. However, they do not provide an indication as to the sequence and location of the DNA binding site. To this end, we used DNase I footprinting techniques to probe for the DNA binding sites of H33258 and 5P2'IBB on the three host duplexes. Figure 4(a) shows the DNase I cleavage profiles resulting from experiments in which the top strand of each duplex (as depicted in Figure 2(a)) was labeled at its 3'-end. We complemented these footprinting studies with corresponding experiments in which the bottom strand of each duplex was labeled at its 3'-end. The DNase I cleavage profiles resulting from these latter studies are shown in Figure S1 of the Supplementary Material. Note that the binding of both H33258 and 5P2'IBB to each of the three host duplexes yields an asymmetric cleavage protection pattern on opposite strands (schematically depicted in Figure 4(b)). This type of cleavage protection pattern has been previously observed in connection with the DNA binding of both minor groove-directed and intercalating agents.^{53–56} The cleavage protection patterns resulting from H33258 and 5P2'IBB binding are consistent with both ligands binding to an identical A₅•T₅ sequence in each of the host duplexes (as denoted by the boxes in Figure 4(b)). The five base pair size and all-A•T composition of the DNA binding site we observe for H33258 are similar to those previously reported for interactions of H33258 with a broad range of DNA duplexes.^{38,42,56–59} Although H33258 and 5P2'IBB bind to an identical A₅•T₅ sequence in the three host duplexes, the location of that sequence differs in each duplex. In this connection, the ligand binding site on the MG2, MG3, and MG4 duplexes is sequentially shifted by one base pair downstream from the site of TOP1-mediated cleavage (see Figure 4(b)). Specifically, the ligand binding sites on the MG2, MG3, and MG4 duplexes encompass the base pairs at positions +3 to +7, +4 to +8, and +5 to +9, respectively.

The optimal binding site on the DNA for stimulation of TOP1-mediated DNA cleavage by H33258 and 5P2'IBB is distal to the cleavage site, and encompasses the base pairs at positions +4 to +8

Our TOP1 poisoning studies described above revealed that both H33258 and 5P2'IBB stimulate TOP1-mediated DNA cleavage of the MG3 duplex to a greater extent than the other two duplexes (Figure 2(b)). Yet the affinities of both ligands for the MG3 duplex are similar to the corresponding affinities they exhibit for the other two duplexes (Table 1). Thus, the increased extent to which both H33258 and 5P2'IBB stimulate TOP1-mediated DNA cleavage of the MG3 duplex relative to the other two duplexes is not the result of an enhanced affinity for that duplex. While both ligands exhibit similar affinities for the MG2 – MG4 duplexes, the location of their binding sites relative to the site of TOP1-mediated DNA cleavage differs for each duplex. Viewed as a whole, these observations suggest that the ligand binding site on the MG3 duplex is optimally located for H33258- and 5P2'IBB-induced stimulation of TOP1-mediated DNA cleavage. Our DNase I footprinting studies indicate that this binding site encompasses the +4 to +8 base pairs. Significantly, a shift in the ligand binding site by one base pair in either direction diminishes the extent to which TOP1-mediated DNA cleavage is stimulated.

The distal location of the optimal ligand binding site relative to the site of DNA cleavage suggests that H33258 and 5P2'IBB poison TOP1 by a different mechanism than CPT and related agents

TOP1 poisoning agents like the camptothecins, the indolocarbazoles, the indenoisoquinolines, and the dibenzonaphthyridines have been shown to stimulate TOP1-mediated DNA cleavage by inserting between the –1 and +1 base pairs and stabilizing the TOP1-DNA cleavable complex through interactions with both the DNA and the enzyme.^{21,34,36} Our results indicate that, unlike these families of TOP1 poisoning agents, H33258 and 5P2'IBB induce optimal poisoning of TOP1 by binding in the DNA minor groove at a site encompassing the +4 to +8

base pairs. Given the distal nature of this binding site relative to the site of cleavage, it is reasonable to suggest that H33258 and 5P2'IBB poison TOP1 via a different molecular mechanism than CPT and related compounds, which bind at the site of cleavage.

One potential mechanism by which H33258, 5P2'IBB, and related minor groove-directed compounds may poison TOP1 is through interactions not only with the DNA, but also with the enzyme. In this connection, Westergaard and coworkers have shown that TOP1 forms a bipartite interaction with DNA.⁶⁰ One of the two DNA regions involved in enzyme binding is downstream from the cleavage site, and encompasses the +6 to +11 base pairs. Minor groove-directed ligands that bind in or near this base pair region may alter the enzyme-DNA interactions in such a way as to stabilize or trap the TOP1-DNA cleavable complex.

A second potential mechanism by which H33258, 5P2'IBB, and related minor groove-directed compounds may poison TOP1 is through the induction of a conformational change in the DNA, which, in turn, stabilizes the TOP1-DNA cleavable complex. One such conformational change might be a bend in the DNA duplex. Appropriately positioned sequence-directed bends in the DNA helix have been implicated in stabilizing the cleavable complex as well as in stimulating enzyme catalysis.^{61,62} In addition, the anthracycline nogalamycin has been shown to poison TOP1 by inducing a bend upstream from the site of cleavage.⁶³ We have shown that minor groove-directed terbenzimidazoles with potent TOP1 poisoning activity preferentially bind and stabilize bent DNA conformations.^{51,52} By contrast minor groove-directed agents like distamycin and netropsin, which are associated with little or no TOP1 poisoning activity,⁴³ exhibit no such binding properties.^{51,52} In fact, distamycin binding removes helical bends from DNA.^{51,64} Taken together, these observations suggest a potential mechanism for drug-induced TOP1 poisoning by the induction of bends in the DNA helix at sites distal (either upstream or downstream) to the site of cleavage. Additional studies are required to assess the veracity of this DNA bending model for ligand-induced poisoning of TOP1.

It should be noted that our results do not exclude the possibility that, in addition to binding in the DNA minor groove downstream from the cleavage site, H33258 and 5P2'IBB may also bind intercalatively at the cleavage site in the presence of TOP1. Such a mode of binding could stimulate TOP1-mediated DNA cleavage, as is the case with CPT. Moreover, this effect could be potentiated by the minor groove-directed binding downstream from the cleavage site. In this connection, a structurally similar analog of H33258 (Hoechst 33342) has been shown to exhibit DNA unwinding properties consistent with a capacity for an intercalative mode of interaction.^{44,49} That said, corresponding studies of 5P2'IBB and related compounds have revealed no evidence for intercalative binding properties.^{46,49} Furthermore, our results indicate that disruption of the A₆•T₆ tract downstream from the site of cleavage abolishes H33258- and 5P2'IBB-induced stimulation of TOP1-mediated cleavage (Figures 2(c) and (d)). These two latter observations would argue against the possibility that the poisoning of TOP1 by H33258 and 5P2'IBB involves binding to the enzyme-DNA interface via intercalation at the site of cleavage.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by grants from the American Cancer Society (RSG-99-153-04-CDD) and NIH (CA097123).

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Abbreviations used

TOP1	topoisomerase I
CPT	camptothecin
H33258	Hoechst 33258
5P2'IBB	5-phenyl-2'-(indolo-6-yl)bibenzimidazole
BSA	bovine serum albumin
SDS	sodium dodecyl sulfate
DTT	DL-dithiothreitol
PMSF	phenylmethanesulfonyl fluoride
EPPS	N-[2-hydroxymethyl]piperazine-N'-[3-propanesulfonic acid]

Figure 1.
Chemical structures of H33258 and 5P2'IBB.

Figure 2.

(a) Sequences of the MG2 – MG4 23mer DNA duplexes. The arrows denote the sites of human TOP1-mediated cleavage induced by H33258 and 5P2'IBB. The 5'-terminal A•T base pair of each A₆•T₆ tract downstream from the cleavage site is highlighted in red. The Arabic numerals denote the number of base pairs downstream from the cleavage site. The asterisks denote the sites of the ³²P labels. All DNA oligonucleotides were obtained in their ion exchange HPLC-purified forms from Integrated DNA Technologies, Inc. (b) Denaturing polyacrylamide gel showing the extent of to which H33258 and 5P2'IBB stimulate human TOP1-mediated cleavage of the MG2 – MG4 duplexes. The marker is a ³²P-3'-end-labeled DNA 17mer, while the arrow indicates the cleavage product. (c) Sequence of the MG3-DisA•T 23mer DNA duplex. The A•T base pairs that are mutated relative to those in MG3 are highlighted in green. The asterisk denotes the site of the ³²P label. (d) Denaturing polyacrylamide gel showing the inability of H33258 and 5P2'IBB to stimulate human TOP1-mediated cleavage of the MG3-DisA•T duplex. In the human TOP1 cleavage assays, single-stranded DNA oligonucleotides were labeled at their 3'-ends with [α -³²P] cordycepin as previously described.⁶⁵ The labeled DNA strands were annealed with their complementary strands in buffer containing 10 mM Tris•HCl (pH 7.8), 100 mM NaCl, and 1 mM EDTA. The annealing process entailed the heating

of the reaction mixture at 95 °C for 5 minutes, followed by slow cooling to room temperature. Duplex DNA substrates (at approximately 100 fmol per reaction), 5 ng of TOP1, and either H33258 or 5P2'IBB at concentration of 1 µM were incubated at room temperature for 30 minutes. The reaction volume was 10 µL and the reaction buffer contained 10 mM Tris•HCl (pH 7.5), 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, 15 µg/mL BSA, and 0.25 mM DTT. The reactions were stopped by addition of SDS to a final concentration of 0.5%. The reaction mixtures were then diluted in 3.3 volumes of buffer containing 98% formamide, 10 mM EDTA, 10 mM NaOH, 1mg/mL xylene cyanol, and 1 mg/mL bromophenol blue. 5 µL of each sample was then loaded onto a denaturing (7 M urea) 16% polyacrylamide gel and electrophoresed at 40 V/cm and 55 °C for 3 hours. The gels were subsequently autoradiographed using Kodak Bio-Max scientific imaging film. Full-length recombinant human DNA TOP1 was expressed in the Bac-to-Bac baculovirus expression system using the protocol provided by the manufacturer (Invitrogen, Carlsbad, CA). The expressed protein was purified by passage over a column of macro-prep ceramic hydroxyapatite type I 80 µm (Bio-Rad, Hercules, CA), and elution with a gradient from 0.2 to 0.8 M potassium phosphate buffer (pH 7.4). The eluted protein was subsequently dialyzed into buffer containing 50% glycerol, 30 mM potassium phosphate (pH 7.0), 0.5 mM EDTA, 2 mM DTT, and 1 mM PMSF and stored at -20 °C. H33258 was obtained from Sigma (St. Louis, MO), while 5P2'IBB was synthesized as previously described.⁴⁶

Figure 3.

Fluorescence profiles for the titration of H33258 (a) and 5P2'IBB (b) with either MG2 (black), MG3 (red), or MG4 (green) at 37 °C. The solid lines reflect the fits of the experimental data with equation (1). The correlation constants (R) resulting from these fits were >0.996 . I_{490} and I_{420} are the fluorescence emission intensities at 490 and 420 nm, respectively. The fluorescence titration experiments were conducted on an AVIV model ATF105 spectrofluorometer (Aviv Biomedical, Lakewood, NJ) equipped with a thermoelectrically controlled cell holder. A quartz cell with a 1 cm path length in both excitation and emission directions was used in all the measurements, with the excitation and emission slit widths being 5 nm. The excitation wavelengths for the H33258 and 5P2'IBB experiments were 355 and 360 nm, respectively. In

each titration, 4 to 12 μL aliquots of 1 μM duplex were sequentially added to ligand solutions that contained either 8 nM 5P2'TBB or 10 nM H33258. These concentrations ensured that each ligand was in its monomeric state.⁶⁶ After each addition, the sample was left to equilibrate for 3 minutes, whereupon the average emission intensity over period of 30 seconds was recorded. Solutions conditions were 10 mM EPPS (pH 7.5), 100 mM KCl, 5 mM MgCl_2 , and 0.1 mM EDTA.

Figure 4.

(a) DNase I footprinting profiles of H33258 and 5P2'IBB binding to the MG2 – MG4 duplexes. In these DNase I footprinting assays, the top strand of each duplex (as depicted in Figure 2(a)) was 3'-end labeled and annealed with its complementary strand as described in the legend to Figure 2. Duplex DNA substrates (at approximately 100 fmol per reaction) and either H33258 or 5P2'IBB at concentration of 1 μ M were incubated at room temperature for 30 minutes. Following this incubation period, 0.2 units of DNase I was added and the samples were further incubated for 5 minutes at room temperature. The reaction volume was 10 μ L and the reaction buffer contained 20 mM Tris•HCl (pH 8.4), 50 mM KCl, and 2 mM MgCl₂. The reactions were stopped by addition of 3.3 volumes of the gel loading buffer described in the legend to

Figure 2. 5 μL of each sample was then loaded onto a denaturing (7 M urea) 16% polyacrylamide gel and electrophoresed at 40 V/cm and 55 $^{\circ}\text{C}$ for 3 hours. The gels were subsequently autoradiographed using Kodak Bio-Max scientific imaging film. G+A sequencing reactions were conducted by combining 3'-end-labeled DNA strands (at approximately 200 fmol per reaction) with salmon testes DNA (at 1 $\mu\text{g}/\mu\text{L}$) and formic acid (at approximately 21%). The reaction mixtures were then incubated at 37 $^{\circ}\text{C}$ for 30 min. The reaction volume was 13 μL and the reaction buffer contained 10 mM Tris•HCl (pH 7.4) and 1 mM EDTA. The reactions were stopped by placement on ice. After the reactions were stopped, 135 μL of distilled water and 15 μL of piperidine were added to each reaction mixture, followed by incubation at 90 $^{\circ}\text{C}$ for 30 min. 200 μL of stop buffer [3.0 M sodium acetate (pH 5.2), 0.5 M EDTA, and 1 $\mu\text{g}/\mu\text{L}$ yeast tRNA], 16 μL of 3.0 M sodium acetate, 1 μL of 1 $\mu\text{g}/\mu\text{L}$ yeast tRNA, and 950 μL of ethanol were then added to each reaction mixture, followed by incubation at -20°C for 15 min. The reaction mixtures were then centrifuged at 4 $^{\circ}\text{C}$ for 15 min. The supernatants were removed and the remaining pellets were air-dried and then resuspended in 30 μL of the gel loading buffer described in the legend to Figure 2. (b) DNase I footprints on both strands of the MG2 – MG4 duplexes induced by the binding of H33258 (blue bars) and 5P2'IBB (green bars). The ligand binding sites on the three duplexes were assigned based on the asymmetric DNase I footprinting model,^{53,54} and are denoted by boxes. The arrows denote the sites of human TOP1-mediated cleavage induced by ligand binding to the boxed sites. The 5'-terminal A•T base pair of each A₆•T₆ tract downstream from the cleavage site is highlighted in red. The asterisks denote the sites of the ³²P labels.

Table 1
Binding Affinities of H33258 and 5P2'IBB for the MG2 – MG4 Duplexes at 37 °C

DNA Duplex	Ligand	K_a (M ⁻¹) ^a
MG2	H33258	$(2.9 \pm 0.1) \times 10^8$
MG3	H33258	$(3.0 \pm 0.1) \times 10^8$
MG4	H33258	$(2.9 \pm 0.1) \times 10^8$
MG2	5P2'IBB	$(3.0 \pm 0.8) \times 10^9$
MG3	5P2'IBB	$(3.0 \pm 0.4) \times 10^9$
MG4	5P2'IBB	$(2.4 \pm 0.6) \times 10^9$

^aLigand-DNA association constants (K_a) were derived from fits of the fluorescence titration profiles shown in Figure 3 with equation (1). The indicated uncertainties reflect the standard deviations of the fitted lines from the experimental data points. The fluorescence titration experiments were conducted as described in the legend to Figure 3. Solutions conditions were 10 mM EPPS (pH 7.5), 100 mM KCl, 5 mM MgCl₂, and 0.1 mM EDTA.