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Ligand Binding to Tandem G Quadruplexes from Human Telomeric DNA

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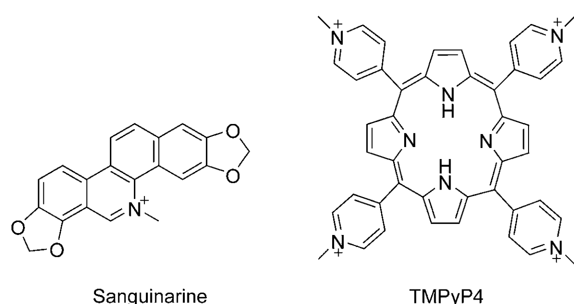
Ligand-induced stabilization of intramolecular telomeric G quadruplexes produced in the single-stranded overhang of the human telomere has become an attractive strategy for the development of anticancer drugs.[1] Several distinct solution conformations of human telomeric G quadruplexes have been elucidated in the presence of sodium[2] and potassium[3] cations. The K^+-form, hybrid-type G-quadruplex structure has been considered to be a physiologically relevant conformation of the human telomeric sequence, and thus, can be specifically targeted by G-quadruplex-interactive, small-molecule drugs.[3a,b] Recently, a beads-on-a-string model was proposed for the telomeric overhang, in which every four consecutive G-rich repeats adopt an individual G-quadruplex structure, and two G-quadruplex units are connected by one TTA linker.[3a,c,4] Ligand binding to the G quadruplex has mostly been investigated on telomere sequences producing a single G quadruplex, but few studies of ligand binding to beads-on-a-string G quadruplexes have been reported. To gain insight into the beads-on-a-string model and the nature of ligand binding, we undertook the polymerase stop assay on human telomere sequences of three to eight repeats (Table S1 in the Supporting Information) with TMPyP4, a G-quadruplex-interactive ligand,[5] and sanguinarine (Scheme 1), a natural isoquinoline alkaloid. The results described in this paper confirm the beads-on-a-string structure of telomeric overhang and suggest a mode of ligand binding between tandem G-quadruplex beads. These observations should be taken into account for structure-based design of anticancer drugs targeting human telomeric DNA.



Scheme 1. Chemical structures of sanguinarine and TMPyP4.

Sanguinarine is a natural isoquinoline alkaloid isolated from the North American herb bloodroot (*Sanguinaria canadensis*). It was approved by the FDA in 2003 to be added to oral cleansing products as an antibacterial agent. Sanguinarine also possesses potent anticancer activity.[6] We have previously reported its DNA-binding activity and distinct sequence selectivity to double-stranded DNA,[7] which was proposed to be one of the molecular mechanisms of its anticancer activity. The structural similarity of sanguinarine with berberine, another isoquinoline alkaloid possessing G-quadruplex-binding activity,[8] prompted us to speculate that sanguinarine is probably a G-quadruplex binder. In this communication, we report its binding to the telomeric overhang using DNA polymerase stop assays.[9] DNA templates Tem-3 and Tem-4 (Table S1), which contains three and four human telomeric repeats d(TTAGGG), respectively, were employed, and TMPyP4 was used as the reference compound in the assay.[5,9a,10] Neither TMPyP4 nor sanguinarine blocked DNA synthesis on Tem-3, because an intramolecular G-quadruplex structure could not form with three human telomeric repeats on Tem-3 (Figure 1). In contrast, both sanguinarine and TMPyP4 produced paused bands in DNA synthesis on Tem-4. The position of the paused bands was the same for the two ligands. For each ligand, a series of concentration-dependent paused bands appeared at the beginning of the G-quadruplex-forming site, that is, the first site of G-rich repeats in Tem-4 (from 3′ to 5′). In the presence of 3 μM TMPyP4, the polymerase reaction was totally suppressed to give no elongation of the primer. The tight binding of sanguinarine and TMPyP4 to the K^+-form hybrid-type G-quadruplex structure was clearly indicated from the large increase in the melting temperature (ΔT_m) of dAGGG(TTAGGG)$_3$ (11.4 °C for sanguinarine and 9.5 °C for TMPyP4 in a 2:1 ligand/DNA molar ratio). Furthermore, a broad negative induced band at 351 nm in the CD spectra confirmed the interaction between sanguinarine and the K^+-form G quadruplex formed by dAGGG(TTAGGG)$_3$ (Figure 2).

Regarding the mode of ligand binding to the G quadruplex, NMR,[9b,10a,11] fibre diffraction,[12] and single-crystal X-ray crystallographic analyses[10b,13] have confirmed that a variety of planar aromatic binders including TMPyP4 interact with the G quadruplex by end stacking rather than intercalation between G tetrads. TMPyP4 was also found to stack onto the external loop of a bimolecular G quadruplex in a crystal structure.[10b] Recently, electrospray ionization time-of-flight mass spectrometry (ESI-TOF-MS) has been used to analyze complexes of G quadruplexes with ligands, since G quadruplexes can maintain their structure in the gas phase in the presence of a suitable cation such as NH_4^+.[14] ESI-MS showed the ammonium ion to be lo-

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cated between the G-tetrads.[15] In addition, ESI-MS in combination with ion-mobility spectrometry and molecular modelling has revealed that end stacking is the dominant binding mode of aromatic planar ligands to G quadruplexes, because end-stacked structures are lower in energy than the intercalated ones.[15a] In the ESI-MS spectra of sanguinarine with dAGGG-(TTAGGG)$_3$ in a 1:1 molar ratio (Figure 3), the complex peaks with two ammonium cations, especially the predominant peaks of $[T4+2NH_4^+ + D]^{4-}$ and $[T4+2NH_4^+ + 2D]^{4-}$, distinctly indicated that two molecules of sanguinarine bind to the G quadruplex by end-stacking, based on the consideration that two ammonium ions are conserved between three tetrads[15a–c] and on the finding that other complexes with stoichiometries greater than 1:2 are, at best, very minor components.

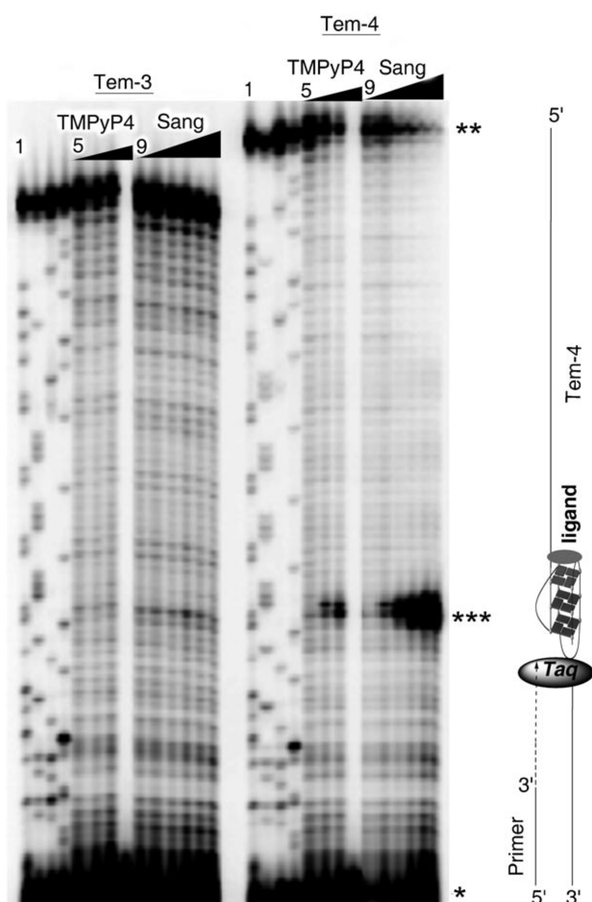

Figure 1. Concentration-dependent inhibition of *Taq* polymerase DNA synthesis by G-quadruplex-binding ligands on DNA templates containing 3 or 4 human telomeric repeats. Lanes 1–4: marker A, C, G and T; lanes 5–8: TMPyP4 (0, 0.3, 1 and 3 μM); lanes 9–14: sanguinarine (0, 0.3, 1, 3, 10 and 30 μM) for each DNA template. * Primer, ** full-length product, *** paused bands. The lane markers represent dideoxy sequencing reactions with the same template as a size marker for the precise arrest sites.[16]

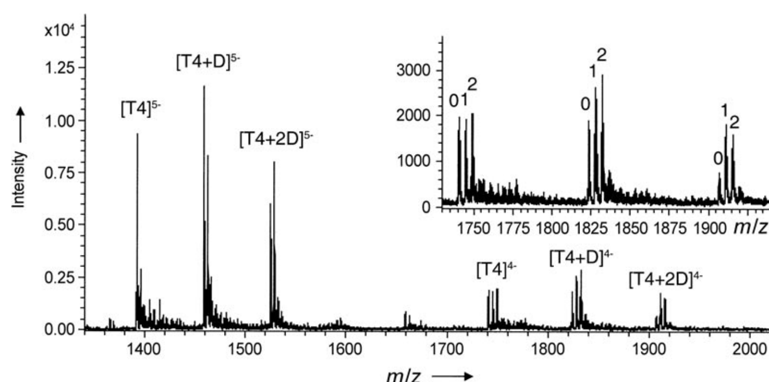

Figure 3. Negative ESI-TOF-MS spectrum of an equimolar mixture of sanguinarine (D, 100 μM) and telomeric sequence dAGGG(TTAGGG)$_3$ (T4, 100 μM). The inset is an enlargement of the distribution of ammonium adducts for the −4 charge state of the G quadruplex and complexes. The numbers represent the number of attached ammonium ions.

The absorption spectra of sanguinarine with dAGGG-(TTAGGG)$_3$ in a 2:1 molar ratio showed a red shift of 10 nm (from 326 nm to 336 nm) associated with 44% hypochromism (Figure 4). These remarkable spectral changes disclosed π–π-stacking interactions between the chromophore of sanguinarine and the G quartet.[10a, 17] On the basis of the characteristic absorption spectral changes and the complex peak of $[T4+2NH_4^+ + 2D]^{4-}$ in the ESI-MS spectrum, sanguinarine most likely binds to the G quadruplex by end stacking, the same

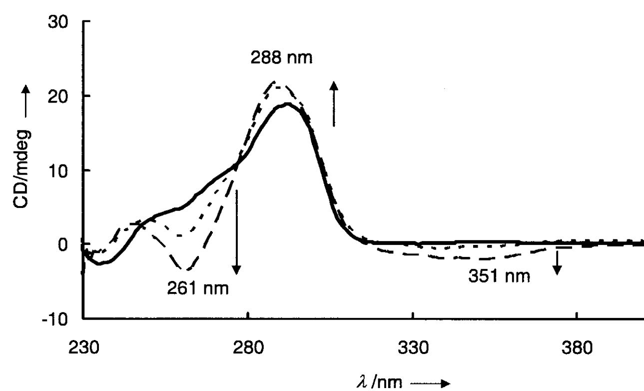

Figure 2. CD spectra of dAGGG(TTAGGG)$_3$ (5 μM) with sanguinarine in 25 mM Tris-HCl buffer (pH 7.0) containing 100 mM KCl. The sanguinarine concentrations were 0 μM (——), 30 μM (····) and 62.5 μM (-----), respectively.

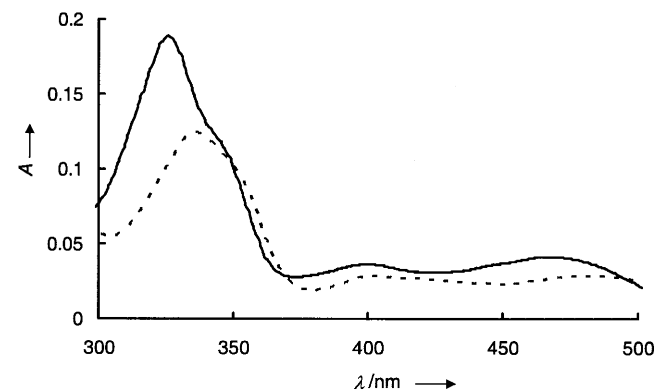

Figure 4. Absorption spectra of sanguinarine (6.0 μM) in the absence (——) or presence of dAGGG(TTAGGG)$_3$ (3.0 μM, ····) in 25 mM Tris-HCl buffer (pH 7.0) containing 100 mM KCl.

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mode of binding of TMPyP4 to a G quadruplex in solution state, which was revealed by NMR.[10a]

To observe the effect of ligand binding to G quadruplex beads and the formation of any characteristic paused bands, we further explored the DNA polymerase stop assay with longer human telomeric DNA (5–8 G-rich repeats, Table S1, Figure 5 A). With Tem-5 as the template, paused bands were detected at two GGG sites: one at the first GGG site and the other at the second GGG site from the 3′ end. Paused bands were not detected at the other three GGG sites. The intensity of the paused bands was slightly stronger for the 3′-side GGG site. These observations were clearly rationalized by the fact that there was an almost equal chance to form a G quadruplex with four out of the five contiguous GGG sites in Tem-5. As the number of GGG sites increased to six in Tem-6 and seven in Tem-7, the formation of paused bands was observed at three and four GGG sites, respectively. The site of paused bands was rationalized by the same argument as for Tem-5. However, a quite different and characteristic pattern of the paused bands appeared on Tem-8. The intensities of the observed five bands were markedly different. The most intense paused band was observed at the first GGG site from the 3′ end. The next most intense band was detected at the fifth GGG site, but its intensity was much weaker than that of the strongest paused band, that is, the first one. Only faint bands were detected at the other three GGG sites. This suggested that DNA with eight G-rich repeats mainly formed four kinds of G quadruplex species, the predominant species composed of two tandem G quadruplexes and three distinct minor species.

An important observation was made in the polymerase stop assay with a small increment of ligand concentration (Figure 5 B). Bands were exclusively produced at the first GGG site when the molar ratio of sanguinarine to Tem-8 was less than or equal to 1:1 (Figure 5 B, lanes 11 and 12 in Tem-8). When

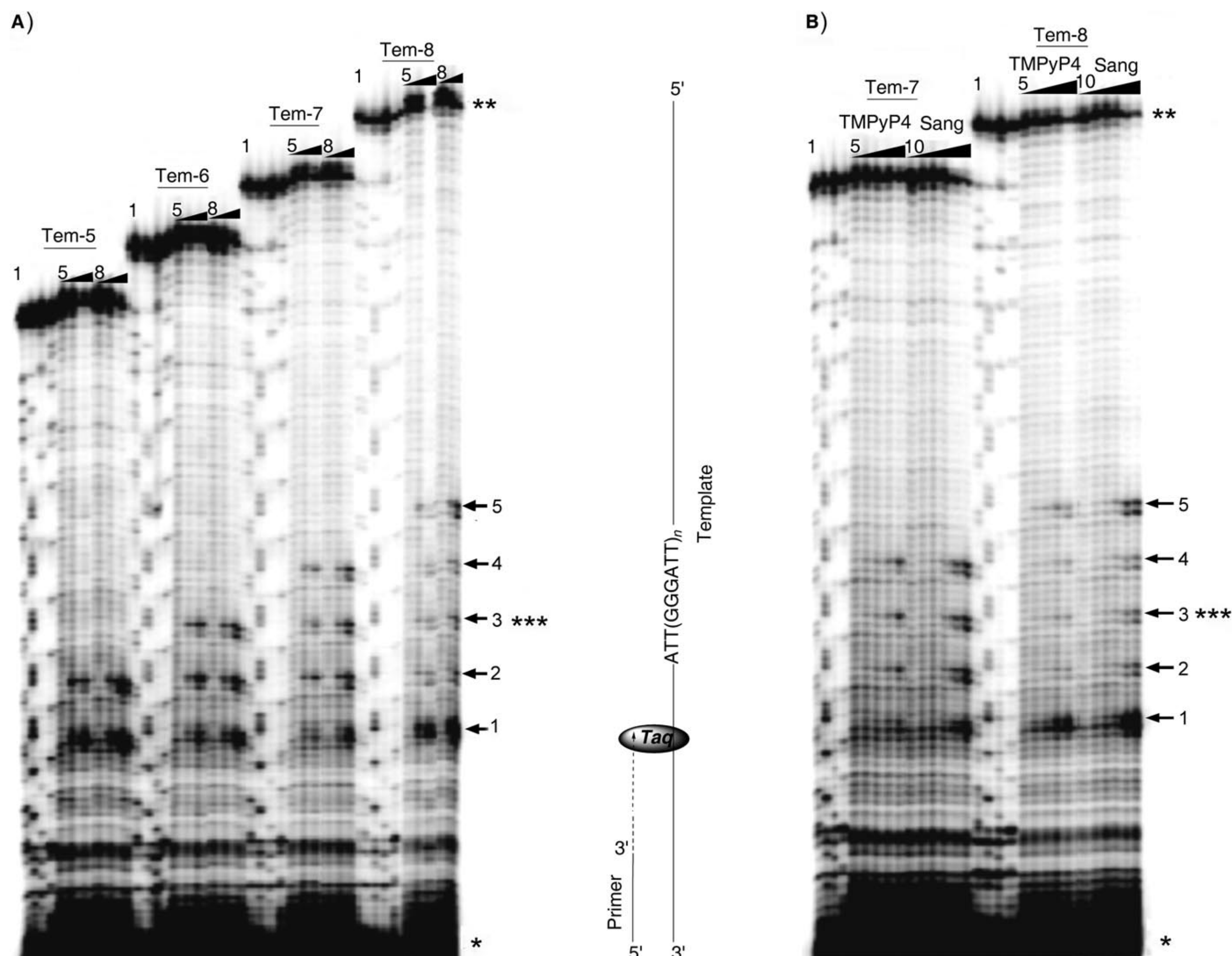

Figure 5. Concentration-dependent inhibition of *Taq* polymerase DNA synthesis by G-quadruplex-binding ligands on DNA templates containing 5 to 8 human telomeric repeats. A) Lanes 1–4: marker A, C, G and T; lanes 5–7: TMPyP4 (0, 0.3 and 1 μM); lanes 8–10: sanguinarine (0, 0.3 and 1 μM) for each DNA template; B) Lanes 1–4: marker A, C, G and T; lanes 5–9: TMPyP4 (0, 0.1, 0.15, 0.3 and 1 μM); lanes 10–15: sanguinarine (0, 0.1, 0.15, 0.3, 1 and 3 μM) for each DNA template. The concentrations of primer and DNA template were 0.1 and 0.15 μM, respectively. * Primer, ** full-length product, *** paused bands. The lane markers represent dideoxy sequencing reactions with the same template as a size marker for the precise arrest sites.[16]

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the sanguinarine/Tem-8 molar ratio increased, a series of paused bands with different intensity appeared. These observations were obtained for both sanguinarine and TMPyP4. The formation of paused bands exclusively at the first GGG site showed that the two-tandem-G-quadruplex structure was the most energetically favourable species among the six possible species existing at a 1:1 molar ratio. It was also apparent from the PAGE analyses that the ligand-bound, two-tandem-G quadruplex was in equilibrium with other minor modes of ligand binding that produced one-bead species with paused bands at every possible G-quadruplex-formation site on Tem-8 under increased ligand concentrations.

ESI-TOF-MS analysis of the complex consisting of sanguinarine and $dAGGG(TTAGGG)_7$ in a 1:1 molar ratio revealed that three molecules of sanguinarine simultaneously bind to the two-tandem-G-quadruplex beads, whereas two molecules of drug externally stack on one bead in both $dAGGG(TTAGGG)_6$ (Figure S1) and $dAGGG(TTAGGG)_3$ (Figure 3). This suggested that one ligand binds between G-quadruplex beads in addition to two ligands binding by end stacking. Additional supporting evidence was supplied from ΔT_m measurements of $dAGGG(TTAGGG)_3$ and $dAGGG(TTAGGG)_7$ (Table 1 and Figure S2) in the presence or absence of sanguinarine. An extra increase of 4.3 °C in the ΔT_m value was observed for $dAGGG(TTAGGG)_7$ compared with that of $dAGGG(TTAGGG)_3$ under the same conditions. The extra increase in the ΔT_m value of $dAGGG(TTAGGG)_7$ might result from a sanguinarine-induced stacking interaction between two G-quadruplex beads in $dAGGG(TTAGGG)_7$.

Table 1. Melting temperatures [°C] of $dAGGG(TTAGGG)_7$ and $dAGGG(TTAGGG)_3$ in the absence (−) or presence (+) of sanguinarine.[a]

Oligodeoxynucleotide	T_m (−)	T_m (+)	ΔT_m
$dAGGG(TTAGGG)_7$	59.9	79.0	19.1
$dAGGG(TTAGGG)_3$	66.8	81.6	14.8

[a] The concentrations of $dAGGG(TTAGGG)_7$, $dAGGG(TTAGGG)_3$ and sanguinarine were 5, 10, and 50 μM, respectively.

In conclusion, the data described here unambiguously confirm the "beads-on-a-string" structure of the human telomeric overhang,[3a,c,4] and suggest a mode of ligand binding between tandem G-quadruplex beads in telomeric DNA. Molecular design focused on the binding of small-molecule drugs between two G-quadruplex beads, which induces the formation of a tightly stacked cluster of G-quadruplex beads, will be a new and effective strategy for structure-based, anticancer drug design targeting human telomeres.

Experimental Section

Materials: Oligodeoxynucleotides used in UV melting, CD and UV absorption were purchased from FASMAC Co., Ltd. (Tokyo, Japan). The 5′ Texas red-labelled DNA primer and human telomeric template (PAGE grade) were obtained from JBioS Co., Ltd. (Saitama, Japan) and dissolved in Milli Q water to be used without further purification. Acrylamide/bisacrylamide solution and ammonium persulfate were purchased from Bio-Rad, and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine was purchased from Fisher. r*Taq* DNA polymerase and dNTPs were purchased from TaKaRa (Shiga, Japan). A sequence marker kit was purchased from Toyobo Co., Ltd. (Osaka, Japan). Stock solutions of sanguinarine and TMPyP4 (1 mM) were made in Milli Q water.

DNA polymerase stop assays: A reaction mixture of template DNA (0.15 μM) and 5′ Texas red-labelled 20-mer primer (0.1 μM) was heated to 95 °C for 3 min in a *Taq* (TaKaRa) reaction buffer (10 mM Tris-HCl, pH 8.30, 50 mM KCl, 1.5 mM $MgCl_2$) and allowed to cool to ambient temperature over 30 min. The requisite amount of ligand was added, and the reaction mixture was incubated at ambient temperature for 1 h prior to the addition of polymerase. *Taq* polymerase and dNTPs were then added to the reaction mixture, which was incubated at 42 °C for 30 min. The reaction products were analyzed by using a Hitachi SQ5500E automated sequencer. The sequence markers were made according to the Toyobo sequence Protocol.[18]

UV melting measurements: Thermal denaturation was performed with a UV-2550 spectrometer (Shimadzu, Japan) equipped with a Shimadzu TMSPC-8 temperature controller. The absorbance was monitored at 295 nm, while the temperature was increased from 4 °C to 98 °C with a heating rate of 1 °C min^{-1}. The DNA concentration was 5 μM for the ΔT_m measurements of $dAGGG(TTAGGG)_3$ in the absence or presence of either sanguinarine or TMPyP4 in a 2:1 ligand/DNA molar ratio. The melting temperature (T_m) is the median of three measurements.

Electrospray mass spectrometry: ESI-TOF-MS experiments were carried out in the negative-ion mode with a Bruker MicrOTOFQ mass spectrometer. Oligodeoxynucleotides $dAGGG(TTAGGG)_3$ (M = 6966.6 Da), $dAGGG(TTAGGG)_6$ (M = 12 694.3 Da) and $dAGGG(TTAGGG)_7$ (M = 14 603.6 Da) were purchased from Invitrogen and used without further purification. The mixture of equimolar sanguinarine and DNA was prepared in aqueous methanol (50 %) containing ammonium acetate (100 mM, pH 7.0). The capillary voltage was +4500 V, and the dry N_2 flow was 5.0 L min^{-1} at 60 °C. Data were analyzed with the software Bruker Daltonics DataAnalysis.

UV absorption spectrometry: Absorption spectra were recorded with a Jasco UV-530 ultraviolet-visible spectrophotometer (Japan) over the spectral range of 200–600 nm.

CD measurements: CD spectra were recorded from 500 to 200 nm with a J-725 spectropolarimeter (Jasco) with a 1 cm path length quartz cuvette at 25 °C. A buffer blank correction was made for all spectra.

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[1] a) S. Neidle, G. Parkinson, *Nat. Rev. Drug Discovery* **2002**, *1*, 383–393; b) L. H. Hurley, *Nat. Rev. Cancer* **2002**, *2*, 188–200; c) J. L. Mergny, J. F. Riou, P. Mailliet, M. P. Teulade-Fichou, E. Gilson, *Nucleic Acids Res.* **2002**, *30*, 839–865.

[2] Y. Wang, D. J. Patel, *Structure* **1993**, *1*, 263–282.

[3] a) A. Ambrus, D. Chen, J. Dai, T. Bialis, R. A. Jones, D. Yang, *Nucleic Acids Res.* **2006**, *34*, 2723–2735; b) K. N. Luu, A. T. Phan, V. Kuryavyi, L. Lacroix, D. J. Patel, *J. Am. Chem. Soc.* **2006**, *128*, 9963–9970; c) Y. Xu, Y. Noguchi, H. Sugiyama, *Bioorg. Med. Chem.* **2006**, *14*, 5584–5591.

[4] a) G. N. Parkinson, M. P. Lee, S. Neidle, *Nature* **2002**, *417*, 876–880; b) H.-Q. Yu, D. Miyoshi, N. Sugimoto, *J. Am. Chem. Soc.* **2006**, *128*, 15461–15468.

[5] a) R. T. Wheelhouse, D. Sun, H. Han, F. X. Han, L. H. Hurley, *J. Am. Chem. Soc.* **1998**, *120*, 3261–3262; b) H. Han, D. R. Langley, A. Rangan, L. H. Hurley, *J. Am. Chem. Soc.* **2001**, *123*, 8902–8913.

[6] a) V. M. Adhami, M. H. Aziz, S. R. Reagan-Shaw, M. Nihal, H. Mukhtar, N. Ahmad, *Mol. Cancer Ther.* **2004**, *3*, 933–940; b) N. Ahmad, S. Gupta, M. M. Husain, K. M. Heiskanen, H. Mukhtar, *Clin. Cancer Res.* **2000**, *6*, 1524–1528; c) S. J. Chmura, M. E. Dolan, A. Cha, H. J. Mauceri, D. W. Kufe, R. R. Weichselbaum, *Clin. Cancer Res.* **2000**, *6*, 737–742.

[7] L.-P. Bai, Z.-Z. Zhao, Z. Cai, Z.-H. Jiang, *Bioorg. Med. Chem.* **2006**, *14*, 5439–5445.

[8] a) M. Franceschin, L. Rossetti, A. D. Ambrosio, S. Schirripa, A. Bianco, G. Ortaggi, M. Savino, C. Schultes, S. Neidle, *Bioorg. Med. Chem. Lett.* **2006**, *16*, 1707–1711; b) W.-J. Zhang, T.-M. Ou, Y.-J. Lu, Y.-Y. Huang, W.-B. Wu, Z.-S. Huang, J.-L. Zhou, K.-Y. Wong, L.-Q. Gu, *Bioorg. Med. Chem.* **2007**, *15*, 5493–5501; c) K. C. Gornall, S. Samosorn, J. Talib, J. B. Bremner, J. L. Beck, *Rapid Commun. Mass Spectrom.* **2007**, *21*, 1759–1766.

[9] a) A. Siddiqui-Jain, C. L. Grand, D. J. Bearss, L. H. Hurley, *Proc. Natl. Acad. Sci. USA* **2002**, *99*, 11593–11598; b) W. Duan, A. Rangan, H. Vankayalapati, M.-Y. Kim, Q. Zeng, D. Sun, H. Han, O. Y. Fedoroff, D. Nishioka, S. Y. Rha, E. Izbicka, D. D. Von Hoff, L. H. Hurley, *Mol. Cancer Ther.* **2001**, *1*, 103–120; c) H. Han, L. H. Hurley, M. Salazar, *Nucleic Acids Res.* **1999**, *27*, 537–542.

[10] a) H. Mita, T. Ohyama, Y. Tanaka, Y. Yamamoto, *Biochemistry* **2006**, *45*, 6765–6772; b) G. N. Parkinson, R. Ghosh, S. Neidle, *Biochemistry* **2007**, *46*, 2390–2397; c) J. Seenisamy, S. Bashyam, V. Gokhale, H. Vankayalapati, D. Sun, A. Siddiqui-Jain, N. Streiner, K. Shin-ya, E. White, W. D. Wilson, L. H. Hurley, *J. Am. Chem. Soc.* **2005**, *127*, 2944–2959.

[11] a) E. Gavathiotis, R. A. Heald, M. F. G. Stevens, M. S. Searle, *Angew. Chem.* **2001**, *113*, 4885–4887; *Angew. Chem. Int. Ed.* **2001**, *40*, 4749–4751; b) A. K. Mehta, Y. Shayo, H. Vankayalapati, L. H. Hurley, J. Schaefer, *Biochemistry* **2004**, *43*, 11953–11958.

[12] M. A. Read, S. Neidle, *Biochemistry* **2000**, *39*, 13422–13432.

[13] a) S. M. Haider, G. N. Parkinson, S. Neidle, *J. Mol. Biol.* **2003**, *326*, 117–125; b) G. R. Clark, P. D. Pytel, C. J. Squire, S. Neidle, *J. Am. Chem. Soc.* **2003**, *125*, 4066–4067.

[14] M. Rueda, F. J. Luque, M. Orozco, *J. Am. Chem. Soc.* **2006**, *128*, 3608–3619.

[15] a) V. Gabelica, E. S. Baker, M.-P. Teulade-Fichou, E. De Pauw, M. T. Bowers, *J. Am. Chem. Soc.* **2007**, *129*, 895–904; b) F. Rosu, V. Gabelica, C. Houssier, P. Colson, E. De Pauw, *Rapid Commun. Mass Spectrom.* **2002**, *16*, 1729–1736; c) F. Rosu, E. De Pauw, L. Guittat, P. Alberti, L. Lacroix, P. Mailliet, J.-F. Riou, J.-L. Mergny, *Biochemistry* **2003**, *42*, 10361–10371; d) J. Zhou, G. Yuan, J. Liu, C.-G. Zhan, *Chem. Eur. J.* **2007**, *13*, 945–949.

[16] D. Sun, K. Guo, B. Rubis, J. J. Rusche, L. H. Hurley, *Nucleic Acids Res.* **2005**, *33*, 6070–6080.

[17] E. Galezowska, A. Masternak, B. Rubis, A. Czyrski, M. Rybczyńska, T. W. Hermann, B. Juskowiak, *Int. J. Biol. Macromol.* **2007**, *41*, 558–563.

[18] M. Hagihara, Y. Goto, K. Nakatani, *ChemBioChem* **2008**, *9*, 510–513.

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