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Regulation of Thrombin Activity by Ligand-Induced Topological Alteration in Thrombin-Binding Aptamer

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Thrombin-binding apatamer (TBA), which forms a G-quadruplex (G4) structure with anti-parallel topology, interacts with thrombin to inhibit its enzymatic activity. Here we show that the G4-topology-altering ligand L2H2-2M2EA-6LCO (6LCO) changes the anti-parallel topology of TBA G4 to parallel topology, thereby abrogating the thrombin-inhibitory activity of TBA. This finding suggests that G4 ligands that alter topology may be promising drug candidates for diseases involving G4-binding proteins.

G-Quadruplex (G4) is a higher-order structure that is formed in single-stranded guanine-rich regions of nucleic acids by stacking of two to four planar G-quartets, each consisting of four guanines linked via Hoogsteen base pairing.[1] G4-forming DNA sequences are widely distributed in the genome, especially in promoter regions of cancer-related genes[2-5] and telomeres.[6,7] They are involved in regulating a wide range of biological phenomena, including oncogene transcription and ribosome biogenesis,[8-11] via their interaction with the corresponding binding proteins.[12-16] G4 mainly adopts three topologies, i.e., parallel, anti-parallel, and hybrid,[17-20] and G4-binding proteins are believed to strictly recognize the G4 topology of their binding partner in cells. For example, HP1α (heterochromatin protein 1α), which plays a role in the maintenance of heterochromatin, binds specifically to the parallel form of telomeric G4,[21] while Rif1 protein, which controls the replication timing in yeast, interacts with parallel and hybrid G4 topology, but not with anti-parallel topology.[22] Furthermore, the enzyme RHAU (RNA helicase associated with AU-rich element), which is a DEAD-box RNA helicase, selectively recognizes and unwinds parallel-type G4s regardless of their sequence.[23,24] In other words, the functions of G4-binding proteins (G4BP) are potentially able to be regulated via G4 topology.

Such restricted recognition between G4 and G4BP has also been utilized in aptamers. For example, the thrombin-binding aptamer (TBA) was identified by SELEX (systematic evolution of ligands by exponential enrichment) screening[25] as a selective binder of thrombin, a serine protease that catalyzes the conversion of fibrinogen to fibrin, leading to blood clotting.[26-30] TBA forms an anti-parallel G4 in which T3 and T12 are precisely oriented with respect to the active site of thrombin, thereby inhibiting thrombin activity (Fig 1A and 1B).[31,32] Clever reported a pyridine-modified TBA and showed that its topology was altered by the addition of Cu^{2+}.[33] The modified TBA without Cu^{2+} inhibited thrombin, whereas the addition of Cu^{2+} abrogated the inhibitory activity by altering the topology of TBA. DeRosa reported that exposure of polyA-polyG-tethered TBA to acidic conditions resulted in the protonation of N1-adenine to form A-G mismatches, which altered the topology of TBA G4, enabling thrombin activity to be controlled by pH change.[34,35] In these strategies, the anti-parallel topology in TBA, which serves as a scaffold for T3 and T12, was altered to cancel the binding to thrombin.[31,32] Ligand-induced topology change, on the other hand, can be applied to unmodified G4s. However, most topology-altering G4 ligands have only been applied to telomeric G4, which can potentially adopt all three topologies.[36-38] In contrast, only a few topology-altering ligands have been reported for other G4s,[39-41] such as TBA G4, which adopt a single topology under the K^+-rich conditions (100–150 mM KCl) commonly used to stabilize G4s.[2-7] Thioflavin T (ThT) induced parallel-type TBA G4, but the topology change only occurred under salt-free conditions,[42] in which thrombin would

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exhibit no activity. Ligand-induced topology change remains challenging in the intracellular environment, where high concentrations of K^+ ions are present.

We have developed a series of polyoxazoles as G4 ligands,[43,44] and found that one of the linear-consecutive hexaoxazole series (6LCOs) selectively induces specific topology of telomeric G4.[45] The G4 ligand L2H2-2M2EA-6LCO (6LCO) was designed based upon the two-point recognition concept, i.e., targeting the G-quartet plane and the groove, and was synthesized as described in our previous report.[45] Here, we show that the enzymatic function of thrombin can be controlled by regulating the binding of thrombin with TBA through the induction of a specific TBA G4 topology with 6LCO (Fig 1C).

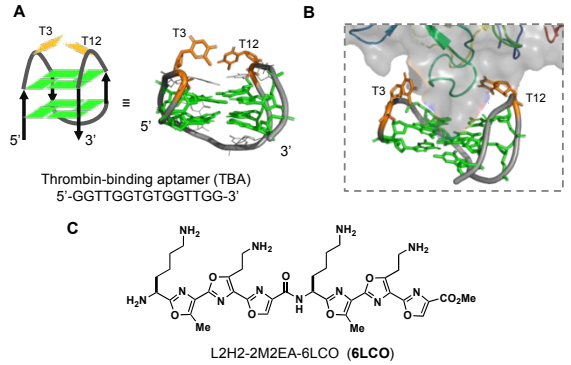

Fig. 1 (A) Schematic model of anti-parallel type G4 in thrombin-binding aptamer (TBA), (B) The structure of the TBA-thrombin complex (PDB:1HAO)[32]. (C) The structure of the ligand L2H2-2M2EA-6LCO (**6LCO**)

Firstly, to establish whether 6LCO could induce a change in the anti-parallel topology of TBA, we analysed its CD spectra (Fig. 2A). As previously reported, TBA G4 showed a positive Cotton effect at 245, 295 nm and a negative one at 270 nm (solid line), indicating the presence of anti-parallel G4, under a K^+-rich condition (10 mM Tris-HCl (pH 7.5) and 100 mM KCl). However, upon the titration of TBA G4 (2 μM) with 6LCO (2–20 μM), the peaks at 245 and 295 nm decreased and that at 260 nm increased suggesting the formation of parallel G4 topology.[46] To support this, we measured the thermal difference spectra (TDS) of TBA in the presence of 6LCO (Fig. 2B, Fig. S1). The UV spectrum of TBA (5 μM) in the presence of 6LCO (10 μM) at 95 °C, where all G4 should be unfolded, was subtracted from the spectrum at 20 °C, where G4 should be fully formed. In TDS, typical G-quadruplex structures exhibit at least two peaks, a positive peak at 270 nm and a negative peak at 295 nm derived from Hoogsteen hydrogen bonding of G-quartets.[47,48] The TDS of a mixture of TBA (5 μM) and 6LCO (0–25 μM) clearly showed both peaks, indicating that TBA forms G4 structure in the presence of 6LCO. Note that in the presence of a high concentration of 6LCO (50 μM), the UV spectrum of TBA below 275 nm was not measurable due to the strong absorbance of 6LCO itself. The CD and TDS spectra both support the view that anti-parallel G4 in TBA was converted into parallel topology G4 by 6LCO.

Since parallel G4 structures have the potential to exist in monomeric as well as dimeric or polymeric forms through G-quartet stacking,[46] we conducted native PAGE analysis (Fig. S2). The band of TBA (1 μM) appeared between 15-mer and 30-mer oligonucleotide markers (lanes 1, 7, and 8), which demonstrates that TBA formed monomeric G4. The complex of TBA (1 μM) and 6LCO (1–10 μM) showed the same mobility (lanes 2–6) as TBA alone, further confirming that TBA G4 bound with 6LCO forms monomeric G4. Taken together, these results indicate that anti-parallel G4 topology of TBA was changed into the monomeric parallel G4 topology in the presence of 6LCO.

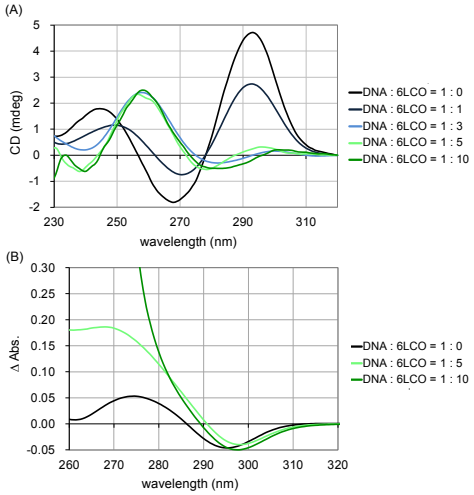

Fig. 2 (A) The CD spectra of TBA (2 μM) in 50 mM Tris-HCl with 100 mM KCl in the presence of 6LCO (2-20 μM). (B) The thermal difference spectra (TDS) of TBA (5 μM) in 50 mM Tris-HCl with 100 mM KCl in the presence of 6LCO (0-50 μM).

In addition, to evaluate the stabilization of TBA-G4 by 6LCO, we carried out FRET (Förster resonance energy transfer) melting analysis[49,50] using dual-labelled TBA (Flu-TBA; Table S1), in which the excitation energy of FAM at the 5'-end is transferred to TAMRA at the 3'-end as long as TBA forms G4 (Fig. S3). Prior to melting analysis, we confirmed by CD analysis that Flu-TBA forms anti-parallel G4 topology and that the addition of 6LCO alters this to parallel topology (Fig. S4). By monitoring the FAM fluorescence during heating, the ΔT_m value of Flu-TBA-G4 was found to be 7.7 °C and the addition of 6LCO increased the T_m value dose-dependently, indicating that 6LCO stabilizes Flu-TBA-G4.

To confirm that the FRET melting results reflected unfolding of TBA G4, CD melting analysis was performed (Fig. S5). The Cotton effect at 295 nm of a solution of TBA (2 μM) alone and that at 260 nm of TBA in the presence of 6LCO (20 μM) were monitored during heating from 20 °C to 90 °C and subsequent annealing to 20 °C (each rate: 1 °C/min). The T_m values of the melting and annealing curves of TBA alone were 53 °C and 49 °C, respectively, whereas those of TBA in the presence of 6LCO were 64 °C and 57 °C, respectively. The greater degree of hysteresis of TBA G4 in the presence of 6LCO, compared with TBA alone, is presumably due to the effect of the ligand on the refolding kinetics of TBA.[51,52] The FRET-melting and CD-melting analyses both indicate that 6LCO thermodynamically stabilizes TBA G4.

To investigate the effect of topological change of TBA G4 by 6LCO on the binding activity with thrombin, we next performed dot-blot experiments. A solution of fluorophore-tagged TBA

(Flu-TBA, 1 μM) and 6LCO was added to a nitrocellulose membrane on which thrombin (5.6 nM) was immobilized by physical adsorption. The membrane was incubated for 30 min, then washed with buffer, and the fluorescence signal on the membrane was scanned. As a result, Flu-TBA remained on the membrane as a strongly fluorescent spot (Fig. 3A, entry 1). In contrast, the addition of 6LCO (1–10 μM) to Flu-TBA decreased the fluorescence in a dose-dependent manner (Fig. 3A, entries 2–6), indicating that thrombin did not interact with TBA G4 in the presence of 6LCO. The fluorescence signal on the membrane was quantified (Fig. 3B) and the 6LCO-induced decrease reached 80% (entry 6; Fig. 3B), compared with Flu-TBA alone. Since 6LCO induces monomeric parallel G4 in TBA, the topological change of TBA by 6LCO inhibits the binding between thrombin and TBA G4.

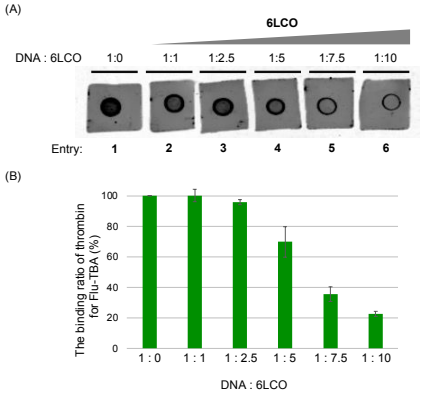

Fig. 3 Inhibition of thrombin-TBA complex formation by 6LCO. (A) The dot-blot experiments with Flu-TBA (0.1 μM) on membrane-immobilized thrombin (5.6 nM/spot) in 50 mM Tris-HCl with 100 mM KCl in the absence (entry 1) or presence of 6LCO (0-1 μM; entry 2-6). (B) The binding ratios of thrombin to Flu-TBA at different DNA : 6LCO ratios were determined from the fluorescence intensities of the blots, quantified using ImageJ. Error bars represent standard derivations of triplicate determinations.

Next, the topological change of TBA G4 by 6LCO was applied to control the thrombin-catalyzed fibrin formation reaction. [33] Thrombin activity was monitored in terms of the formation of insoluble fibrin by turbidimetry at 550 nm and the $t_{1/2}$, which is defined as the time required to reach 50% fibrin formation, was calculated (Fig. 4). In the presence of thrombin (8.9 nM), fibrinogen (8 mg/mL) was rapidly transformed to fibrin and the $t_{1/2}$ was 69 sec. In the presence of TBA (890 nM), which binds to the active site of thrombin with anti-parallel topology and inhibits the activity, the formation of fibrin was drastically decreased and the $t_{1/2}$ reached 2,100 sec. In contrast, the addition of TBA together with 6LCO (4.5, 8.9 μM) decreased the inhibitory activity of TBA. Since no inhibitory activity was observed in the presence of 6LCO without TBA (4.5 μM: $t_{1/2}$ = 73 sec, 8.9 μM: $t_{1/2}$ = 76 sec), thrombin did not interact directly with 6LCO. These results suggest that the binding of TBA to thrombin is inhibited by the 6LCO-induced topological change of TBA G4 to parallel topology, wherein T3 and T12 of TBA, which are required for binding to the active site of thrombin, [31,32] are located in the propeller loops, so that TBA can no longer inhibit thrombin.

To gain deeper insights into the mechanism by which 6LCO induces the formation of a parallel topology G4 in TBA, we conducted docking simulations to analyze the binding modes of 6LCO with both anti-parallel and parallel TBA G4 structures. Since no reported parallel-type TBA G4 structure was available, we first generated a molecular model of the parallel TBA G4 by incorporating all guanines involved in G-quartet formation in the original anti-parallel structure (see supplemental methods). Following the acquisition of the initial coordinates of 6LCO, we performed docking simulations separately on the calculated parallel-type TBA G4 and the anti-parallel-type TBA G4 (PDB: 1C32)[53], and acquired 100 docking poses with the lowest docking scores (Fig. 5A). The lowest docking score poses of 6LCO with the parallel and anti-parallel topologies of TBA G4 are depicted in Fig. 5B,C and Fig. S6). The results suggest that 6LCO binds preferentially to the parallel topology G4 in TBA.

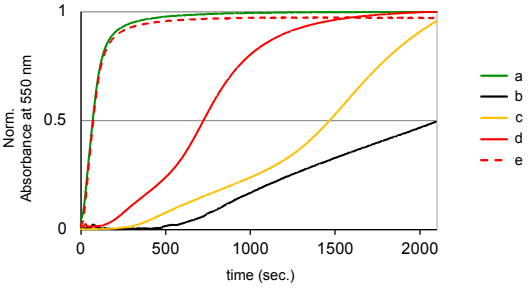

Fig.4 Time-dependent fibrin formation by thrombin in 50 mM Tris-HCl with 100 mM KCl. In every experiment, thrombin (8.9 nM) and fibrinogen (8 mg/mL) were used. The conditions were as follows: a) thrombin + fibrinogen, b) thrombin + TBA (890 nM) + fibrinogen, c) thrombin + TBA (890 nM) + 6LCO (4.5 μM) + fibrinogen, d) thrombin + TBA (890 nM) + 6LCO (9.0 μM) + fibrinogen, e) thrombin + 6LCO (8.9 μM) + fibrinogen.

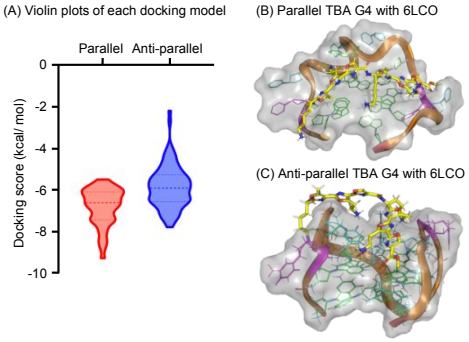

Fig. 5 Docking models of TBA G4 with 6LCO; A) The violin plot of the docking score of each pose (Red: parallel TBA G4 with 6LCO, blue: anti-parallel TBA G4 with 6LCO), B) parallel TBA G4 with 6LCO, C) anti-parallel TBA G4 with 6LCO (G-quartet plane: green, T3 and T12: magenta, ligand: yellow).

Conclusions

Our results show that inhibition of the enzymatic activity of thrombin by TBA-G4 is blocked by the G4-topology-altering ligand L2H2-2M2EA-6LCO (6LCO), which switches the anti-parallel form of G4 in TBA to parallel topology. Although the molecular basis of the dynamic refolding mechanism of the TBA G4 by 6LCO is still unclear, topology-altering G4 ligands such as 6LCO should be useful tools to elucidate the functions of G4 and G4BP. For example, since 6LCO altered the topology of TBA G4 even under physiological conditions (pH 7.4, 100–150 mM KCl), this would allow the function of G4BPs to be controlled by conformational switching of G4 inside the cell. Recently, G4

ligands that alter the conformation of nucleobases in the loop region in G4 without inducing topological change have been reported to control the activity of G4BPs.[54,55] Thus, both conformation-modulating and topology-altering ligands might be promising drug candidates for diseases involving G4BPs.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

1 J. T. Davis, *Angew. Chem. Int. Ed.,* 2004, **43**, 668-698.
2 A. Bugaut, S. Balasubramanian, *Nucleic Acids Res*., 2012, **40**, 4727-4741.
3 A. Verma,V. K. Yadav, R. Basundra, A. Kumar, S. Chowdhury, *Nucleic Acids Res.*, 2009, **37**, 4194-4204.
4 J. L. Huppert, A. Bugaut, S.Kumari, S. Balasubramanian, *Nucleic Acids Res*., 2008, **36**, 6260-6268.
5 J. L. Huppert, S. Balasubramanian, *Nucleic Acids Res*., 2007, **35**, 406-413.
6 H. Seimiya, *Cancer Sci.*, 2020, **111**, 3089-3099.
7 T. M. Bryan, *Molecules*, 2020, **25**, 3686.
8 L. A. Cahoon, H. S. Seifert, *Science*, 2009, **325**, 764-767.
9 S. Kumari, A. Bugaut, J. L. Huppert, S. Balasubramanian, *Nature Chem. Biol.*, 2007, **3**, 218-221.
10 A. Siddiqui-Jain, C. L. Grand, D. J. Bearss, L. H. Hurley, *Proc. Natl. Acad. Sci. U. S. A.* 2002, **99**, 11593-11598.
11 V. González, K. Guo, L. H. Hurley, D. Sun, *J. Biol. Chem.*, 2009, **284**, 23622-23635.
12 G. Wu, Z. Xing, E. J. Tran, D. Yang, *Proc. Natl. Acad. Sci. U. S. A.,* 2019, **116**, 20453-20461.
13 S. Cogoi, M. Paramasivam, A. Membrino, K. K. Yokoyama, L. E. Xodo, *J. Biol. Chem.*, 2010, **285**, 22003-22016.
14 A. C. Krüger, M. K. Raarup, M. M. Nielsen, M. Kristensen, F. Besenbacher, J. Kjems, V. Birkedal, *Eur. Biophys. J.*, 2010, **39**, 1343-1350.
15 G. Biffi, D. Tannahill, S. Balasubramanian, *J. Am. Chem. Soc*., 2012, **134**, 11974-11976.
16 S. Ray, J. N. Bandaria, M. H. Qureshi, A. Yildiz, H. Balci, *Proc. Natl. Acad. Sci. U. S. A.,* 2014, **111**, 2990-2995.
17 Y. Wang,; D. J. Patel, *Structure*, 1993, **1**, 263-282.
18 B. Heddi, A. T. Phan, *J. Am. Chem. Soc.*, 2011, **133**, 9824-9833.
19 A. Ambrus, D. Chen, J. Dai, T. Bialis, R. A. Jones, D. Yang, *Nucleic Acids Res.*, 2006, **34**, 2723-2735.
20 G. N. Parkinson, M. P. H .Lee, S. Neidle, *Nature*, 2002, **417**, 876-880.
21 R. J. Roach, M. Garavís, C. González, G. B. Jameson, V. V. Filichev, T. K. Hale, *Nucleic Acids Res.,* 2020, **48**, 682-693.
22 H. Masai, R. Fukatsu, N. Kakusho, Y. Kanoh, K. Moriyama,Y. Ma, K. Iida, K. Nagasawa, *Sci. Rep.,* 2019, **9**, 8618.
23 N. M. Gueddouda, O. Mendoza, D. Gomez, A. Bourdoncle, J.-L. Mergny, *Biochim. Biophys. Acta*, 2017, **1861**, 1382-1388.
24 B. Heddi, V. V. Cheong, H. Martadinata, A. T. Phan, *Proc. Natl. Acad. Sci. U. S. A.*, 2015, **112**, 9608-9613.
25 L. C. Bock, L. C. Griffin, J. A. Latham, E. H. Vermaas, J. J. Toole, *Nature*, 1992, **355**, 564-566.
26 J. W. Fenton, *Ann. N. Y. Acad. Sci.,* 1981, **370**, 468-495.
27 M. A. Shuman, *Ann. N. Y. Acad. Sci.,* 1986, **485**, 228-239.
28 R. F. Macaya, P. Schultze, F. W. Smith, J. A. Roe, J. Feigon, *Proc. Natl. Acad. Sci. USA,* 1993, **90**, 3745-3749.
29 I. R. Krauss, A. Merlino, A. Randazzo, E. Novellino, L. Mazzarella, F. Sica, *Nucl. Acids Res.,* 2012, **40**, 8119-8128.
30 C. Riccardi, E. Napolitano, C. Platella, D. Musumeci, D. Montesarchio, *Pharmacol. Ther.,* 2021, **217**, 107649.
31 K. Padmanabhan, K. P. Padmanabhan, J. D. Ferrara, J. E. Sadler, A. Tulinsky, *J. Biol. Chem.,* 1993, **268**, 17651-17654.
32 K. Padmanabhan, A. Tulinsky, *Acta. Cryst.*, 1996, **52**, 272-282.
33 D. M. Engelhard, J. Nowack, G. H. Clever, *Angew. Chem. Int. Ed.,* 2017, **56**, 11640-11644.
34 M. Belleperche, M. C. DeRosa, *Pharmaceuticals*, 2018, **11**, 80.
35 E. M. McConnell, R. Bolzon, P. Mezin, G. Frahm, M. Johnston, M. C. DeRosa, *Bioconjug. Chem*., 2016, **27**, 1493-1499.
36 Y. Ma, K. Iida, K. Nagasawa, *Biochem. Biophys. Res. Commun.*, 2020, **531**, 3-17.
37 S. Asamitsu, T. Bando, H. Sugiyama, *Chem. Eur. J.,* 2019, **25**, 417-430.
38 M. P. O'Hagan, J. C. Morales, M. C. Galan, *Eur. J. Org. Chem*., 2019, **31-32**, 4995-5017.
39 E. Napolitano, C. Riccardi, R. Gaglione, A. Arciello, V. Pirota, A. Triveri, F. Doria, D. Musumeci, D. Montesarchio, *Int. J. Biol. Macromol.,* 2023, **224**, 344-357.
40 A. T. T. Tu, K. Hoshi, Y. Ma, T. Oyama, S. Suzuki, K. Tsukakoshi, K. Nagasawa, K. Ikebukuro, T. Yamazaki, *ACS Chem. Biol.,* 2022, **17**, 1703-1713.
41 A. Biswas, S. B. Singh, C. S. Todankar, S. Sudhakar, S. P. P. Pany, P. I. Pradeepkumar, *Phys. Chem. Chem. Phys.*, 2022, **24**, 6238-6255.
42 D. Zhao, X. Dong, N. Jiang, D. Zhang, C. Liu, *Nucleic Acids Res.*, 2014, **42**, 11612-11621.
43 M. Tera, H. Ishizuka, M. Takagi, M. Suganuma, K. Shin-Ya, K. Nagasawa, *Angew. Chem. Int. Ed.*, 2008, **47**, 5557-5560.
44 K. Iida, K. Nagasawa, *Chem. Rec.*, 2013, **13**, 539-548.
45 S. Sasaki, Y. Ma, T. Ishizuka, H. L. Bao, T. Hirokawa, Y. Xu, M. Tera, K. Nagasawa, *RSC Adv.,* 2020, **10**, 43319-43323.
46 C.-F. Tang, R. H. Shafer, *J. Am. Chem. Soc.,* 2006, **128**, 5966-5973.
47 J.-L. Mergny, J. Li, L. Lacroix, S. Amrane, J. B. Chaires, *Nucl. Acids Res.,* 2005, **33**, e138.
48 A. I. Karsisiotis, N. M. Hessari, E. Novellino, G. P. Spada, A. Randazzo, M. W. da Silva, *Angew. Chem. Int. Ed.,*2011, **123**, 10833.
49 A. De Cian, L. Guittat, M. Kaiser, B. Saccà, S. Amrane, A. Bourdoncle, P. Alberti, M.-P. Teulade-Fichou, L. Lacroix, J. L. Mergny, *Methods*, 2007, **42**, 183-195.
50 A. De Rache, J. L. Mergny, *Biochimie*, 2015, **115**, 194-202.
51 H.-Q. Yu, D. Miyoshi, N. Sugimoto, *J. Am. Chem. Soc.,* 2006, **128**, 15461-15468.
52 J.-L. Mergny, L. Lacroix, *Oligonucleotides,* 2003, **13**, 515-537.
53 V. M. Marathias, P. H. Bolton, *Nucl. Acids Res*., 2000, **28**, 1969-1977.
54 B.-C. Zhu, J. He, X.-Y. Xia, J. Jiang, W. Liu, L.-Y. Liu, B.-B. Liang, H.-G. Yao, Z. Ke, W. Xia, Z.-W. Mao, *Chem. Sci.,* 2022, **13**, 8371-8379.
55 R. Ishikawa, M. Yasuda, S. Sasaki, Y. Ma, K. Nagasawa, M. Tera, *Chem. Commun.,* 2021, **57**, 7236-7239.

