ChemComm

Stabilization of telomeric G-quadruplex by ligand binding increases susceptibility to S1 nuclease

Journal:	*ChemComm*
Manuscript ID	CC-COM-06-2021-003294.R1
Article Type:	Communication

COMMUNICATION

Stabilization of telomeric G-quadruplex by ligand binding increases susceptibility to S1 nuclease

Received 00th January 20xx,
Accepted 00th January 20xx

DOI: 10.1039/x0xx00000x

Ryo Ishikawa,[a,+] Mizuho Yasuda, [a,+] Shogo Sasaki, [a,+] Yue Ma,[b] Kazuo Nagasawa*[a] and Masayuki Tera*[a]

The extent of thermodynamic stabilization of telomeric G-quadruplex (G4) by isomers of G4 ligand L2H2-6OTD, a telomestatin analog, is inversely correlated with susceptibility to S1 nuclease. L2H2-6OTD facilitated the S1 nuclease activities through the base flipping in G4, unlike the conventional role of G4 ligands which inhibit the protein binding to DNA/RNA upon ligand interactions.

G-Quadruplexes (G4s) are higher-order nucleic acid structures that can be formed in guanine-rich regions, such as gene promoters[1, 2] and telomeres in DNA,[3, 4] and in untranslated regions in RNA.[5, 6] Although G4 exhibits high thermodynamic stability, there is believed to be a dynamic equilibrium between G4 and the duplex with the complementary strand, and this equilibrium is involved in DNA transcription,[7] replication,[8, 9] and cellular senescence,[4, 10, 11] together with DNA-binding proteins such as nucleases[12, 13] and helicases.[14-18] Telomeres, which have a long single-stranded region in the T-loop with no complementary strand, provide a favorable environment for G4 formation, and have been intensively studied for the past two decades. For example, the helicase RTEL1 unwinds the telomeric T-loop structure to maintain telomere integrity, but stabilization of telomeric G4 by the G4 ligand TMPyP4 decreases the RTEL1 activity.[19] Pif1 preferentially unfolds telomeric G4, and its efficacy depends on its topology and reaction conditions.[20] The exonuclease EXO1 resolves telomeric G4 at the replication fork to enable faithful replication of telomeres,[21] and this is interrupted by G4 ligands. Thus, the bulky structure of G4 is generally more resistant to nuclease or helicase activity compared with the canonical duplex or single-stranded nucleic acid structures. Furthermore, G4 ligands bind either to the G-quartet or to loops, and thermodynamically stabilize G4. Consequently, G4 ligands have been intensively studied as candidate anti-cancer agents.

In contrast, however, Hurley and co-workers reported that the G4 ligand telomestatin bound to telomeric G4 and thereby enhanced the activity of S1 nuclease.[22, 23] Thus, the relationship between stabilization of G4s and susceptibility to nuclease/helicase activity remains to be fully elucidated.

We previously developed the telomestatin analog L2H2-6OTD, which is a G-quartet binder that strongly stabilizes G4 through both π-π interaction with G-quartet and electrostatic interaction with the phosphate backbone of G4.[24] In this study, we investigated the relationships among thermodynamic stabilization of telomeric G4 by its ligands, S1 nuclease kinetics, and cleavage sites in ligand-stabilized telomeric G4, employing three isomers of L2H2-6OTD, namely, 3,3-, 4,2-, 5,1-6OTD (**1**–**3**) with different oxazole connectivity (Fig. 1a). These 6OTDs have a macrocyclic hexaoxazole structure that stacks onto G-quartet, but they show different extents of thermodynamic stabilization of G4s due to their different side chain directions.[25, 26] We have shown that these 6OTDs stabilize telomeric G4 with ΔT_m values of 4.1, 7.9, and 18.8 ˚C, respectively (ΔT_m values: **1** > **2** > **3**).[25]

We first measured the CD spectra to clarify the topology of telomeric G4 stabilized by **1**–**3**, because telomeric G4 forms various topologies (i.e. parallel,[27] anti-parallel,[28] and hybrid[29-31]), depending on the buffer conditions. HT24, a well-characterized hybrid form of telomeric G4 (Table S1), was used in this study.[30] From the CD spectra, HT24 formed hybrid G4 in the presence of 100 mM KCl (negative Cotton effect around 240 nm and positive Cotton effect around 265 and 295 nm) and the hybrid topology was predominantly retained in the presence of **1**–**3** (Fig 1b).[32] Thus, **1**–**3** stabilized hybrid-type telomeric G4 with various ΔT_m values.

[a] *Department of Biotechnology and Life Science, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588 (Japan).*
[b] *Institute of Global Innovation Research, Tokyo University of Agriculture and Technology, 2-24-16 Naka-cho, Koganei, Tokyo 184-8588 (Japan).*

† Electronic Supplementary Information (ESI) available: [details of any supplementary information available should be included here]. See DOI: 10.1039/x0xx00000x

[+] These authors contributed equally to this work.

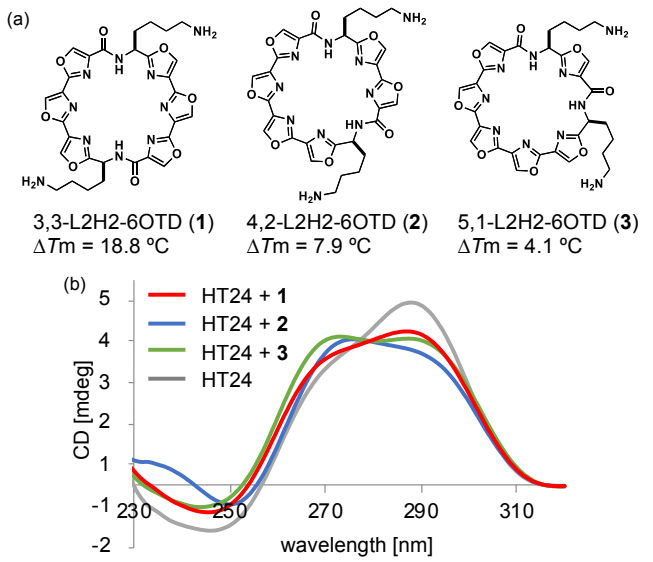

Fig. 1 (a) Structures of 3,3-, 4,2-, 5,1-L2H2-6OTD (**1**–**3**) and ΔT_m values for telomeric G4 with **1**–**3** obtained by FRET-melting analysis. (b) CD spectra of HT24 (10 μM) in Tris-KCl buffer in the presence or absence of **1**-**3** (100 μM).

S1 nuclease, a single-strand-specific nuclease, is widely used to probe higher-order nucleic acid structure, because its activity strongly depends on the substrate structure.[33] We then tested S1 nuclease activity on HT24 in the presence or absence of L2H2-6OTDs to clarify the effects of the ligands on the susceptibility to S1 nuclease activity. From the time-course analysis of S1 nuclease activity, T_{50} (the time required for 50% digestion of oligonucleotide) for HT24 (2 μM) was determined as 14.2 min, which is much longer than that of single-stranded oligonucleotide (ss24: <1 min) (Fig. 2, S1 and S2). We also confirmed that the G4 structures were preserved under the acidic (pH 5.1) S1 nuclease reaction conditions (Fig. S3a).[34] These results indicate that G4 is not favorable as a substrate of S1 nuclease. In contrast, the addition of **1**–**3** (10 μM) accelerated the S1 nuclease reaction, with T_{50} values of 2.8, 5.1, and 10.1 min. These times are inversely related to the extents of thermodynamic G4 stabilization of **1**–**3** under both physiological and S1 nuclease reaction conditions (Fig. S3b) (rank order of T_{50}: **3** > **2** > **1** and T_m: **1** > **2** > **3**). We next examined the S1 nuclease reaction on SS24 in the presence or absence of the G4 ligands, but observed no kinetics enhancement (Fig. S2). These results suggest that the S1 nuclease reaction is promoted by the formation of ligand-G4 complex.

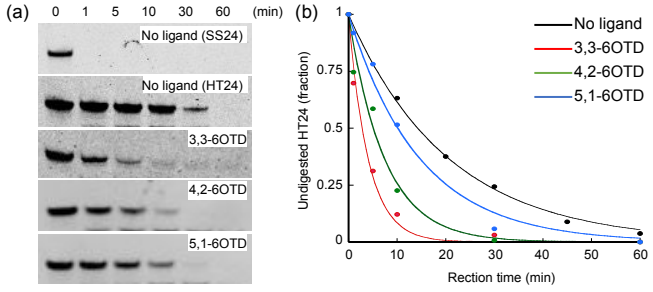

Fig. 2 (a) Time-course of remaining HT24 and SS24 (2 μM) after S1 nuclease treatment in the presence of **1**–**3** (20 μM). The gels were stained with SYBR-gold to visualize oligonucleotides. (b) Analysis of first-order kinetics of S1 nuclease reaction using remaining HT24 bands quantified by Image J.

In order to address the reason for this, we characterized the S1 nuclease digest of G4 stabilized by **1** (Fig. 3), which showed the greatest kinetic enhancement among the tested ligands. The digestion products of HT24 by S1 nuclease in the presence of **1** were resolved by denaturing PAGE and the resulting four major bands were analyzed by both denaturing PAGE (Fig. S4) and MALDI-MS (Fig. 3 c-f). Band a was characterized as HT24, band b as the 19-mer from the 5'-end (HT19), band c as the 13-mer from the 5'-end, and band d as the 11-mer from 3'-end. Thus, S1 nuclease cleaved between A and T in loops 2 and 3 (Fig. 3 c-f). At a lower concentration of 3,3-6OTD (4 μM), S1 nuclease yielded only HT19 (Fig. 3b), indicating that 3,3-6OTD promotes S1 nuclease cleavage predominantly at loop 3, then loop 2 in HT24.

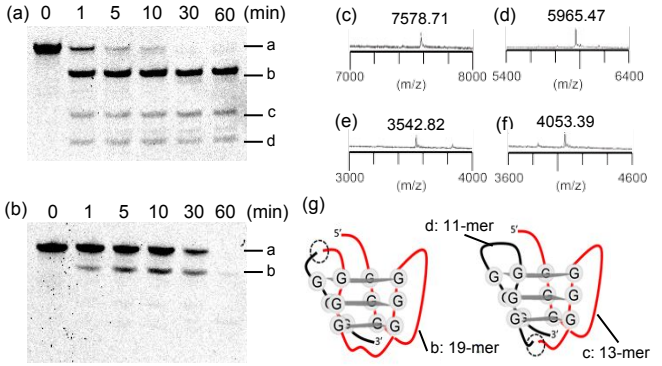

Fig. 3 Denaturing PAGE of S1 nuclease digests of HT24 in the presence of (a) 3,3-6OTD (20 μM) and (b) 3,3-6OTD (4 μM). Four bands were detected by staining with SYBR-gold. MALDI-MS analysis of S1-digested HT24: (c) band a: HT24 (calcd: 7575.15), (d) band b: 19-mer of 5'-TTGGGTTAGGGTTAGGGTT (calcd: 5961.05), (e) band c: 13-mer of 5'-TTGGGTTAGGGTT (calcd: 4051.77), and band d: 11-mer of 5'P-AGGGTTAGGGA (calcd: 3541.37). (f) Schematic illustration of the structure of HT 24 G4 and characterization of bands b, c, and d.

2-Aminopurine (2-AP), an adenine analogue, shows fluorescence at 380 nm, and can be used to probe the conformation of the G4 loop, because the 2-AP emission is drastically quenched near the G4 quartet, but recovers through a flipping out process.[35,36] We utilized 2-AP-modified HT24 (table S1) to examine whether 3,3-6OTD binds to site 1 or 2 in the telomeric G4 (Fig. 4). When HT24-20AP, which has 2-AP substitution in the loop 3 adenine, was titrated with 3,3-6OTD, the fluorescence of 2-AP recovered dose-dependently (Fig. 4). This could be due to binding of 3,3-6OTD at site 1 in the telomeric G4, causing 2-AP in loop 3, located near site 1, to be displaced from over the G-quartet (Fig. 4b).[37] Thus, the plot of the fluorescence recovery ratio versus 3,3-6OTD concentration should give the dissociation constant of 3,3-6OTD from site 1, and this was calculated as 83 nM. HT24-14AP having loop 2 adenine substitution also showed a fluorescent increment upon addition of 3,3-6OTD, and the Kd value of site 2 was 643 nM. In contrast, 2-AP in loop 1 (HT24-8AP) was more quenched by the addition of 3,3-6OTD (Fig. S5). The ligand binding might make the 2-AP locate near the electron rich guanine, which could potentially quench 2-AP fluorescence. These results indicate that 3,3-6OTD binds preferentially to site 1.

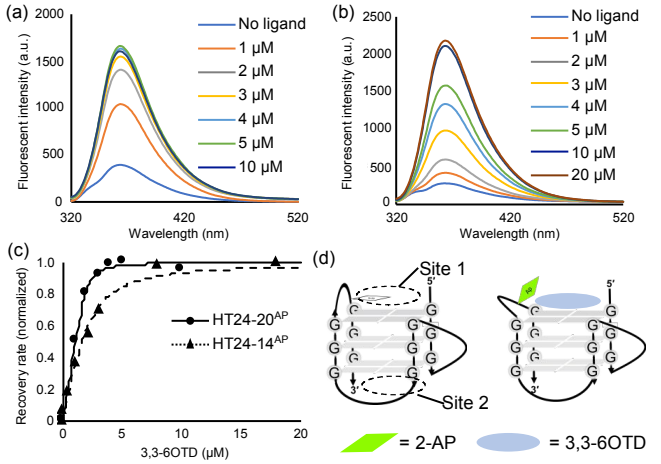

Fig. 4 3,3-6OTD titration of (a) HT24-20AP and (b) HT24-14AP in Tris-K buffer. (c) Plot of fluorescence recovery rate (circle: HT24-20AP or triangle: HT24-14AP) versus concentration of 3,3-6OTD. (d) Schematic illustration of the structural change of HT24-20AP upon the addition of 3,3-OTD. The Kd values were calculated by non-linear regression fitting.

Hydrolysis of single-stranded oligonucleotide by S1 nuclease requires the recognition of both the nucleobase and phosphodiester bond at the cleavage site.[38] NMR analysis has shown that adenine located in loop 3 of HT24 is flipped out from over the 5'-end G-quartet (site 1) by 3,3-6OTD (Fig. 4) thereby highly wobbled,[39] and this corresponds to the main cleavage site of S1 nuclease (Fig. 3). Being stable G-quartet structure, the guanine bases attributing the G4 are highly tolerated from enzymatic attack.[23] Thus, 3,3-6OTD induced flipping out of the adenine in loop 3, allowing the exposed adenine base to be efficiently recognized by S1 nuclease. Since the base stacking between G-quartet and its next bases stabilizes G4, the flipping out of adenines in the loops 2 and 3 could thermodynamically destabilize the G4. However, the complete overlapping between 6OTD and G-quartet should compensate the G4 stability and replace the adenine over the G-quartet.[39] The cleavage efficiency at loop 2 was lower than that at loop 3 (Fig. 3), which could be explained by finding that the dissociation constant of 3,3-6OTD for site 2 near loop 2 is higher than that of site 1 (Fig. 4), so that exposure of adenine in loop 2 is promoted less effectively.

Interestingly, HT19 generated by cleavage at loop 3 in HT24 was highly resistant to hydrolysis by S1 nuclease in the presence of 3,3-6OTD (Fig. 3a). The CD spectrum of HT19 suggested the formation of a G-triplex that would be further stabilized by 3,3-6OTD, as demonstrated by UV-melting analysis at 295 nm, based on the absorbance due to Hoogsteen hydrogen bonding derived from G-triplex (Fig S6).[40]

G4 ligands generally stabilize G4 thermodynamically, and their biological activity has been discussed on the premise that the biological activity of G4 ligands relies on stable G4-ligand complexation. In other word, G4 ligands have been thought to reduce the susceptibility of G4 to nuclease, helicase, and polymerase activities. Indeed, in the case of DNase 1, an endonuclease, its activity towards telomeric G4 was inhibited by formation of the complex G4-3,3-6OTD (Fig. S7). In this study, we used S1 nuclease and telomeric G4 as a model system and found that G4-ligand complexation enhances nuclease activity towards G4. This may suggest that the biological activity of G4 ligands arises through facilitation of G4-related protein recognition by ligand-G4 interactions. In this model system, double-stranded telomeric sequence, which is most abundant in the cellular environment, served no nuclease enhancement by the addition of 3,3-6OTD (Fig. S8 and S9), while the smeared bands by S1 nuclease treatment might contain secondary DNA structures such as G4 and i-motif.[41] This result suggested that such nuclease enhancement requires G4-ligand complexation, which could be inhibited by the double-strand formation even under the acidic (pH 5.1) condition.[42] Our results also suggest that S1 nuclease could be used as a reporter to investigate the exposure of nucleobases from the G4 loop in the presence of ligands. This approach could be applied to the analysis of G4-ligand interactions in combination with conventional DMS foot-printing analysis.

L2H2-6OTDs (**1**–**3**) not only thermodynamically stabilize telomeric hybrid G4 but also render it more susceptible to S1 nuclease activity. By means of S1 nuclease treatment, MALDI-MS, and ligand titration with 2-AP-modified G4, we found that 3,3-6OTD flips the adenine base out in the telomeric G4 loop, thereby enabling S1 nuclease to access its cleavage sites.

This research was partially funded by JSPS/MEXT KAKENHI (JP19K05743, JP21H00275 to M.T.; JP20H02876, JP18H04387 to K.N.; JP20K15411 to Y.M.; JP20J13814 to S.S.), AMED JP20wm0325016, Inamori Foundation, Tokuyama Science Foundation, Asahi Glass Foundation to M.T., and JST ACT-X JPMJAX191E to Y.M. This work was inspired by the international and interdisciplinary environment of the JSPS Asian CORE Program of ACBI (Asian Chemical Biology Initiative).

Conflicts of interest

There are no conflicts to declare.

Notes and references

1 V. S. Chambers, G. Marsico, J. M. Boutell, M. Di Antonio, G. P. Smith and S. Balasubramanian, *Nat. Biotechnol.*, 2015, **33**, 877-881.
2 D. Rhodes and H. J. Lipps, *Nucleic Acids Res.*, 2015, **43**, 8627-8637.
3 S. Neidle, *FEBS J.*, 2010, **277**, 1118-1125.
4 H. Seimiya, *Cancer Sci.*, 2020, **111**, 3089-3099.
5 A. Arora and B. Suess, *RNA Biol.*, 2011, **8**, 802-805.
6 D. Varshney, J. Spiegel, K. Zyner, D. Tannahill and S. Balasubramanian, *Nat. Rev. Mol. Cell Biol.*, 2020, **21**, 459-474.
7 A. Siddiqui-Jain, C. L. Grand, D. J. Bearss and L. H. Hurley, *Proc. Natl. Acad. Sci. U. S. A.*, 2002, **99**, 11593-11598.
8 Y. Kanoh, S. Matsumoto, R. Fukatsu, N. Kakusho, N. Kono, C. Renard-Guillet, K. Masuda, K. Iida, K. Nagasawa, K. Shirahige and H. Masai, *Nat. Struct. Mol. Biol.*, 2015, **22**, 889-897.
9 K. Paeschke, J. A. Capra and V. A. Zakian, *Cell*, 2011, **145**, 678-691.
10 H. Han and L. H. Hurley, *Trends Pharmacol. Sci*, 2000, **21**, 136-142.
11 S. M. Kerwin, *Curr. Pharm. Des.*, 2000, **6**, 441-478.
12 Z. Liu and W. Gilbert, *Cell*, 1994, **77**, 1083-1092.
13 A. C. Vallur and N. Maizels, *Proc. Natl. Acad. Sci. U. S. A.*, 2008, **105**, 16508-16512.

14 H. Sun, J. K. Karow, I. D. Hickson and N. Maizels, *J. Biol. Chem.*, 1998, **273**, 27587-27592.
15 J. P. Vaughn, S. D. Creacy, E. D. Routh, C. Joyner-Butt, G. S. Jenkins, S. Pauli, Y. Nagamine and S. A. Akman, *J. Biol. Chem.*, 2005, **280**, 38117-38120.
16 Y. Wu, K. Shin-ya and R. M. Brosh, Jr., *Mol. Cell. Biol.*, 2008, **28**, 4116-4128.
17 C. Ribeyre, J. Lopes, J. B. Boulé, A. Piazza, A. Guédin, V. A. Zakian, J. L. Mergny and A. Nicolas, *PLoS Genet.*, 2009, **5**, e1000475.
18 N. M. Gueddouda, O. Mendoza, D. Gomez, A. Bourdoncle and J. L. Mergny, *Biochim. Biophys. Acta. Gen. Subj.*, 2017, **1861**, 1382-1388.
19 J. B. Vannier, V. Pavicic-Kaltenbrunner, M. I. Petalcorin, H. Ding and S. J. Boulton, *Cell*, 2012, **149**, 795-806.
20 A. K. Byrd, M. R. Bell and K. D. Raney, *J. Biol. Chem.*, 2018, **293**, 17792-17802.
21 S. Stroik, K. Kurtz, K. Lin, S. Karachenets, C. L. Myers, A. K. Bielinsky and E. A. Hendrickson, *Nucleic Acids Res.*, 2020, **48**, 4960-4975.
22 O. L. Acevedo, L. A. Dickinson, T. J. Macke and C. A. Thomas, Jr., *Nucleic Acids Res.*, 1991, **19**, 3409-3419.
23 M. Y. Kim, M. Gleason-Guzman, E. Izbicka, D. Nishioka and L. H. Hurley, *Cancer Res.*, 2003, **63**, 3247-3256.
24 K. Iida and K. Nagasawa, *Chem. Rec.*, 2013, **13**, 539-548.
25 M. Sakuma, Y. Ma, Y. Tsushima, K. Iida, T. Hirokawa and K. Nagasawa, *Org. Biomol. Chem.*, 2016, **14**, 5109-5116.
26 M. Tera, H. Ishizuka, M. Takagi, M. Suganuma, K. Shin-ya and K. Nagasawa, *Angew. Chem. Int. Ed. Engl.*, 2008, **47**, 5557-5560.
27 B. Heddi and A. T. Phan, *J. Am. Chem. Soc.*, 2011, **133**, 9824-9833.
28 Y. Wang and D. J. Patel, *Structure*, 1993, **1**, 263-282.
29 A. Ambrus, D. Chen, J. Dai, T. Bialis, R. A. Jones and D. Yang, *Nucleic Acids Res.*, 2006, **34**, 2723-2735.
30 K. N. Luu, A. T. Phan, V. Kuryavyi, L. Lacroix and D. J. Patel, *J. Am. Chem. Soc.*, 2006, **128**, 9963-9970.
31 Y. Xu, Y. Noguchi and H. Sugiyama, *Bioorg. Med. Chem.*, 2006, **14**, 5584-5591.
32 R. Del Villar-Guerra, J. O. Trent and J. B. Chaires, *Angew. Chem. Int. Ed. Engl.*, 2018, **57**, 7171-7175.
33 Z. Zhou, J. Zhu, L. Zhang, Y. Du, S. Dong and E. Wang, *Anal. Chem.*, 2013, **85**, 2431-2435.
34 P. Bucek, J. Jaumot, A. Aviñó, R. Eritja and R. Gargallo, *Chem. Eur. J.*, 2009, **15**, 12663-12671.
35 R. D. Gray, L. Petraccone, R. Buscaglia and J. B. Chaires, *Methods Mol. Biol.*, 2010, **608**, 121-136.
36 S. Sasaki, Y. Ma, T. Ishizuka, H. L. Bao, T. Hirokawa, Y. Xu, M. Tera and K. Nagasawa, *Rsc Adv.*, 2020, **10**, 43319-43323.
37 T. D. Bradrick and J. P. Marino, *Rna*, 2004, **10**, 1459-1468.
38 T. Kovaľ, L. H. Østergaard, J. Lehmbeck, A. Nørgaard, P. Lipovová, J. Dušková, T. Skálová, M. Trundová, P. Kolenko, K. Fejfarová, J. Stránský, L. Švecová, J. Hašek and J. Dohnálek, *PLoS One*, 2016, **11**, e0168832.
39 W. J. Chung, B. Heddi, M. Tera, K. Iida, K. Nagasawa and A. T. Phan, *J. Am. Chem. Soc.*, 2013, **135**, 13495-13501.
40 D. Koirala, T. Mashimo, Y. Sannohe, Z. Yu, H. Mao and H. Sugiyama, *Chem. Commun.*, 2012, **48**, 2006-2008.
41 The excess amount of S1 nuclease non-specifically digested double-stranded DNA, however no reaction enhancement was observed by the addition of **1**.
42 A.T. Phan, J.L. Mergny, *Nucleic Acids Res.*, 2002, **30**, 4618–4625.