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Title: Novel cyclic naphthalene diimide dimer with strengthened ability to stabilize dimeric G-quadruplex

Authors: Shigeori Takenaka, Ryusuke Takeuchi, Tingting Zou, Daiki Wakahara, Yoshifumi Nakano, and Shinobu Sato

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COMMUNICATION

Novel cyclic naphthalene diimide dimer with strengthened ability to stabilize dimeric G-quadruplex

Ryusuke Takeuchi,[a] Tingting Zou,[ab] Daiki Wakahara,[a] Yoshifumi Nakano,[a] Shinobu Sato,[ab] Shigeori Takenaka [ab*]

Abstract: New type of dimeric cyclic naphthalene diimide derivatives (cNDI-dimers) carrying varied linker length were designed and synthesized to recognize dimeric G-quadruplex structures. All of the cNDI-dimers exhibited high preference to recognize G-quadruplex structures, and significant enhanced the thermal stability of dimeric G-quadruplex structure than cNDI monomer with increasing melting temperature by more than 23 ºC, which indicated the strengthened ability of cNDI dimers for stabilizing dimeric G-quadruplex. cNDI dimers also performed stronger ability to inhibit telomerase activity and stop telomere DNA elongation than cNDI monomer, which showed an improved anti-cancer potentiality for further therapeutic application.

Telomerase was thought to be essential for the immortalization of human cells, for its function in restoring telomeric DNA sequences[1]. It has been reported that telomerase was up-regulated in 85% of human cancer cells[2,3], and its mutant with complete-activity inhibition could lead to the death of tumour cells[4]. Besides, telomerase is generally not expressed in most normal human cells. These critical features lead to the rapid development of telomerase -targeted cancer therapies in advanced clinical trials[5–7].

Over past two decades, four stranded G-quadruplex nucleic acid structure is of great interest as a potential therapeutic target[8,9], for the important roles it played in regulating gene expression and even translation[10]. It also has been proved that G-quadruplex structures formed in G-rich telomeric DNA can inhibit telomerase activity[11–13]. For this reason, it's considered as a promising therapeutic path to develop small molecules, working as G4 ligands, to selectively stabilize human telomeric DNA with four stranded G-quadruplex structures, further enhance the inhibition of telomerase activity and induce cancer cell death[14]. Besides, some reports already suggested that G-quadruplex structure in telomeric sequence assembled like beads on a string[15,16], G4 ligands which can selectively target multiple G4 repeats might be promising for further enhanced specificity on inhibiting telomerase function, and are attracting more attention of G-quadruplex researchers. These several years, some ligand-dimers [17-24] and tetramer [25] have been reported with strong ability to recognize dimeric or multiple G-quadruplex structures, which supported that G4 ligand-multimer might be a good model for stabilizing G-quadruplex repeats.

As one important G4 ligand, naphthalene diimide can recognize G-quadruplex structure through π-π stacking[26], and its binding affinity to G-quadruplex was rarely affected even under in vivo mimic molecular crowding condition and may represent superior telomerase inhibitors in cell nuclei[27,28]. Our group has been working on designing novel G4 ligands with improved specificity for recognizing G-quadruplex[29-31], and previously we reported cyclic naphthalene diimide coupling with cyclohexane (cNDI-ch), which exhibited significant higher affinity (260-fold) towards binding with G-quadruplex than double strand DNA[29]. Besides, Chiara Marchetti et al also reported similar macrocyclic naphthalene diimides coupling with phenyl ring, which exhibited high selectivity for G-quadruplex structure and showed anti-proliferative effects on cancer line with low range of concentrations [32]. Here in this study, we report a group of cNDI-dimers by linking two cNDI monomers with alkyl chain, and these cNDI-dimers could recognize dimeric G-quadruplex structure and show enhanced ability to stabilize G-quadruplex structures.

Based on our previous reports[29], cNDI coupling with glutamine acid was synthesized as a cNDI-monomer, which contains a free amino group, and enable higher flexibility for constructing new derivatives. Then two cNDI monomers (**1**) was linked by different length of alkyl chain (n=3, 5 or 7) to obtain a group of cNDI dimers

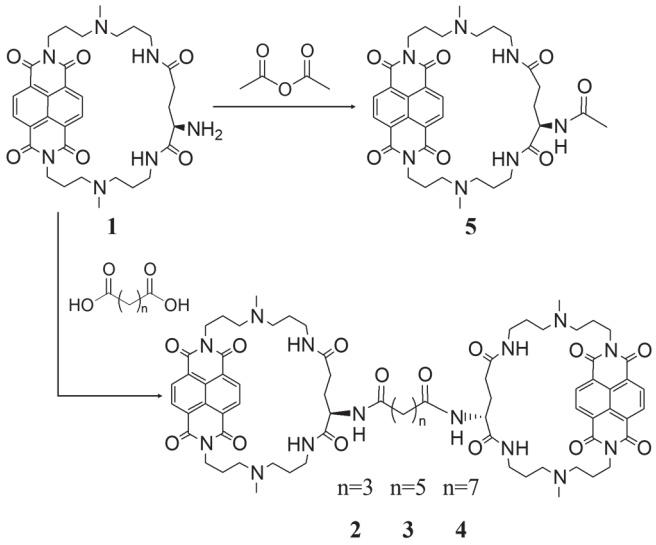

Figure 1. Chemical structures of monomeric cNDI (**1**), cNDI-dimers (**2-4**) with varied linker chain (n=3, 5, or 7) and cNDI-Ac (**5**).

[a] R. Takeuchi, Dr. T. Zou, D. Wakahara, Y. Nakano, Dr. S. Sato, Prof. S. Takenaka
Department of Applied Chemistry, Kyushu Institute of Technology, Fukuoka 804-8550, Japan
shige@che.kyutech.ac.jp
[b] Dr. T. Zou, Dr. S. Sato, Prof. S. Takenaka
Research Center for Bio-microsensing Technology, Kyushu Institute of Technology, Fukuoka 804-8550, Japan

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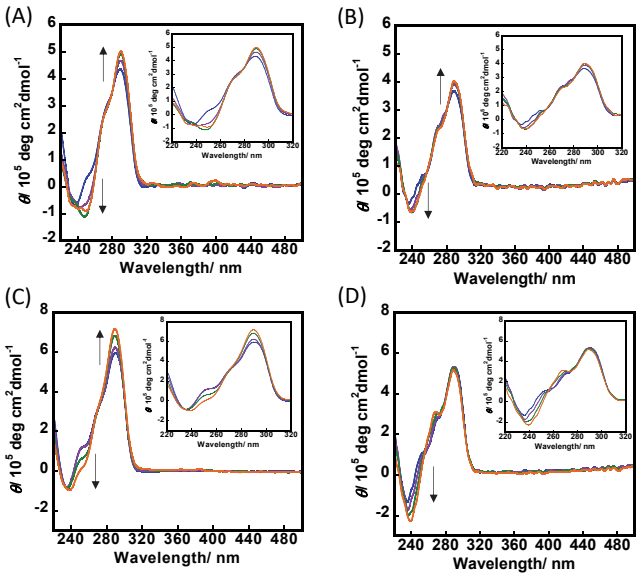

Figure 2. CD spectra analysis. G1 (1.5 μM) with the presence of varying equivalents of **3** (A), and **5** (B); G2T1 (1.5 μM) with the presence of **3** (C), and **5** (D). Buffer: 50 mM Tris-HCl (pH 7.4) and 100 mM KCl. Ligand vs. DNA; 0:1 (Blue); 1:1 (Purple); 2:1 (Green); 3:1 (Orange).

(**2-4**, Fig. 1). To avoid the influence of free amino group when comparing the performance of cNDI-dimers and cNDI-monomer, in this research, the free amino group was protected by acyl group, and the obtained cNDI-Ac (**5**) was adopted as the monomer control (Fig. 1). The synthesis process and chemical confirmation of **2-5** was detailed clarified in the supplementary information.

Two telomeric sequences, G1, 5'-TA(GGGTTA)$_3$-GGG-3', and G2T1, 5'-A(TTAGGG)$_8$-3', were adopted as G-quadruplex model, which contained one hybrid G4 structure, and two G4 units respectively[25]. Within the presence of K^+, G-quadruplex structures were prepared by a slow-cool down annealing process. Under circular dichroism (CD) spectra measurement, a typical spectrum of hybrid G4 structure with a negative band at 240 nm and a positive band at 290 nm (Fig. 2 and Fig. S10) was verified. Similarly to previous reported NDI derivatives[33,34], as titrating **2-4** or **5** to G1, the clear intensity shifting of negative band at 240 nm (weakened) and positive bands at 290 nm (enhanced) were observed, which revealed the G4 structures could be recognized by these cNDI derivatives. As titrating **2-4** or **5** to G2T1, decreased negative bands were observed for all ligands, but comparing to **5**, all three cNDI-dimers showed more obvious enhanced positive band (Fig. 2D), which supported that cNDI-dimers preferred a stronger ability to recognize dimeric G-quadruplex structures. To be mentioned, adding cNDI-dimer to G1 induced slightly right shift of the negative band (Fig. 2B), which might suggest that the free naphthalene diimide skeleton of cNDI-dimers may interact with G-quadruplex and induce some structure change.

To evaluate the binding affinity of ligands towards G4 structure, we employed Isothermal Titration Calorimetry (ITC) for determination. As a powerful technique, ITC can provide the binding affinity of ligand to G-quadruplex, and also help elucidate the thermodynamic change during recognition[35,36]. The binding affinities of **2-4** and **5** towards G-quadruplex DNA (G1, G2T1) and double-strand DNA (HP27 oligo sequence, 5'-GCGATTCTCGGCTTTGCCGAGAATCGC-3') were investigated. According to the results (Table 1, Fig. S11-12), **2-4** and **5** showed similarly high affinity towards binding to single G-quadruplex (over 2×10^6 M^{-1}), and cNDI-dimers' affinity were slightly higher than **5**. However, when towards recognizing dimeric G-quadruplexes, **2-4** exhibited decreased binding affinity (around 3×10^5 M^{-1}), and lower than that of **5** towards dimeric G-quadruplex. One considerable reason was that higher ΔS of **2-4** binding with dimeric intramolecular G-quadruplex (resulting higher ΔG) may partly reflect inherent differences in hydration for DNA structure and/or binding–induced release of counterions [37,38]. And another possible reason was that the bigger structure of cNDI-dimer may decrease its accessibility to dimeric G-quadruplex [39]. Although **3** showed slight stronger affinity towards G-quadruplex than **2** and **4**, the difference was not significant. Besides, for both **2-4** and **5**, the energy changes towards recognizing HP27 were quite small, and no clear binding affinity fitting curve could be obtained, which further indicated the enhanced ability of cNDI derivatives to discriminate G-quadruplex and duplex DNA (Fig. S13).

When comparing the thermodynamic parameter changes, **2-4** and **5** binding to G-quadruplex structures were exothermic, but **2-4** induced larger negative ΔH and ΔS values than **5**, which suggested that cNDI-dimer-G-quadruplex complex was more stable than cNDI-Ac-G-quadruplex complex. UV-Vis was also adopted for confirming the binding performance of these cNDI derivatives (Fig. S14). When titrating annealed G1 or G2T1 sequence to **2** or **5**, large hypochromic, small red shifts and a single isosbestic point were obtained at 390 nm for all titrations, suggesting the stacking interaction between naphthalene diimide

Table 1. Binding affinity of **2-5** with G1 and G2T1.

	G1				G2T1			
	2	**3**	**4**	**5**	**2**	**3**	**4**	**5**
K_a / 10^5 M^{-1}	41.9±0.8	44.9±2.0	38.6±1.3	21.2±0.1	2.80±0.01	3.8±0.02	3.1 ±0.00	17.2±0.1
n	1	1	1	2	1	1	1	3
ΔH / kcal mol^{-1}	-11.8±0.06	-13.4±0.1	-13.8±0.2	-7.7±0.50	-11.3±0.07	-11.5±0.2	-11.4±0.2	-8.7±0.04
$-T\Delta S$ / kcal mol^{-1}	2.76±0.07	4.31±0.11	4.79±0.09	-0.92±0.54	3.88±0.18	3.88±0.22	3.93±0.06	0.06±0.04
ΔS / cal mol^{-1} K^{-1}	-9.25±0.25	-14.5±0.38	-16.1±0.31	3.08±1.81	-13.0±0.59	-13.0±0.73	-13.2±0.22	-0.19±0.14
ΔG / kcal mol^{-1}	-9.0±0.2	-9.1±0.03	-9.0±0.03	-8.6±0.04	-7.4±0.01	-7.6±0.02	-7.5±0.04	-8.5±0.01

Condition: 50 mM Potassium phosphate buffer (pH 7.0), 25ºC.

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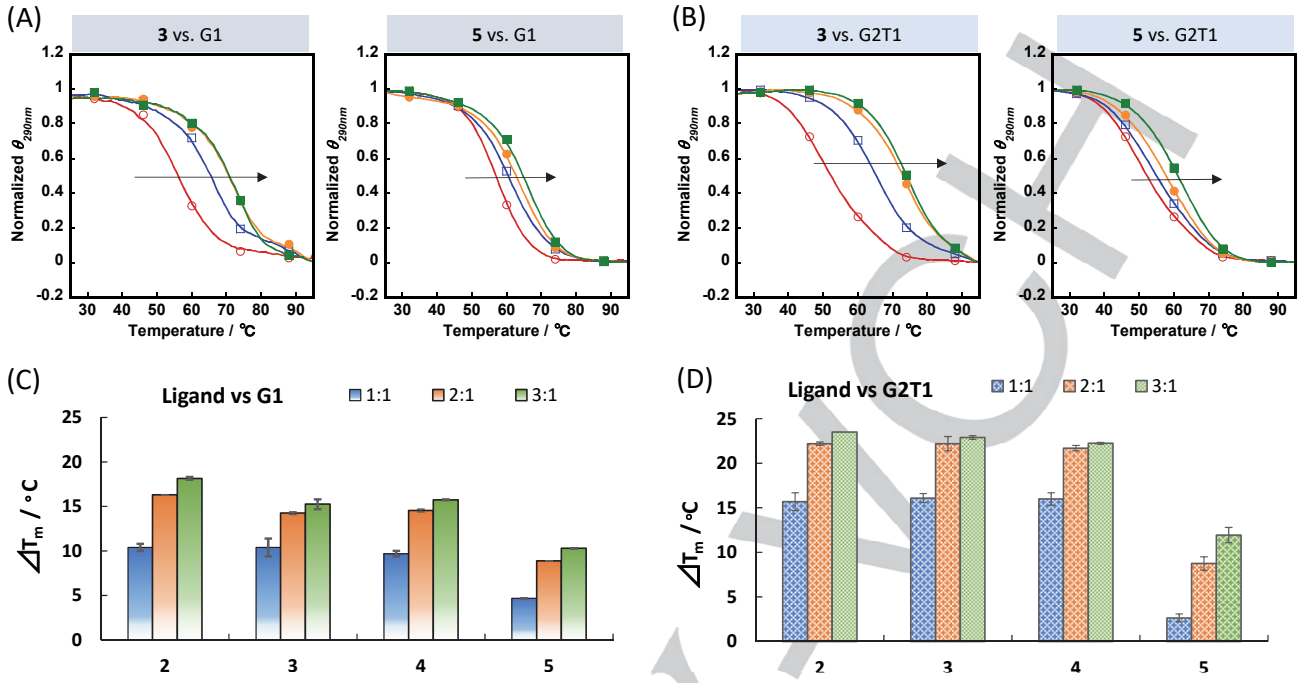

Figure 3. Melting curves of 1.5 μM G1 (A), and 1.5 μM G2T1 (B) with the presence of varying equivalents of **3** (left), and **5** (right); ΔTm plot of G1 (C), and G2T1 (D) in the presence of varying equivalents of **2-5**. Buffer: 50 mM Tris-HCl (pH 7.4) and 30 mM KCl. Ligand vs. DNA; 0:1 (Red); 1:1 (Blue); 2:1 (Orange); 3:1 (Green).

skeleton and G-quartet planes as a main driving force in their interaction, and formed a unified G-quadruplex-ligand complex structure.

After adding cNDI derivatives, the melting temperature change (ΔT_m) was calculated, which represent the ability of cNDI derivatives for stabilizing G-quadruplex structure. For HP27, almost no enhancement were observed for both cNDI-Ac and cNDI-dimers (Fig. S15). As adding **5** to G1 in 2:1 ratio, T_m increased by 8.9 ºC, which is similar to the case of **2-4** to G1 in 1:1 ratio (T_m was increased by 9.7-10.4 ºC) (Fig. 3A and 3C, Fig S16). As previously mentioned, when **2-4** recognizing the single G-quadruplex, one naphthalene diimide skeleton stacked to G-quartet, another free naphthalene diimide might form some interaction with G-quadruplex structure, and further enhance the stabilization, which also might be correlated with the observed CD spectra shift. However, **5** showed much weaker ability to stabilize G2T1 structure in 1:1 ratio with increasing the T_m only by 2.7±0.6 ºC, and continuing adding **5** to G2T1 to 2:1 ratio, T_m was increased by 8.8±1.1 ºC (T_m was 59.9 ºC). While, when adding cNDI-dimers to G2T1 in 1:1 ratio, T_m increased by 15.7-16.1 ºC (enhanced to 67.0-67.4 ºC), which is even much higher than adding **5** in 2:1 ratio. These results also suggested that cNDI-dimer-G-quadruplex complex was more stable, which was in consistent with the thermodynamic parameters obtained by ITC, and further confirmed the stronger ability of **2-4** for stabilizing dimeric G-quadruplex structure (Fig. 3B and 3D, Fig. S16). Regarding the possible binding models, when adding **5** to G2T1 in 2:1 ratio, naphthalene diimide skeletons may stabilize single G-quadruplex structure or two G-quadruplex structures, as for **2-4**, the preference for recognizing the dimeric G-quadruplex structure strengthened their ability to enhance the thermal stability of G2T1. G4 structures were significantly stabilized when adding cNDI-dimers to G2T1 in 2:1 ratio (T_m increased by 21.7-22.2 ºC), which further convinced the high ability of cNDI-dimers for stabilizing G-quadruplex structure, continuously adding **2-4** didn't further increase the melting temperature supported that cNDI-dimers' preference for simultaneously recognizing two G4 units (Fig. 3, Fig S16). In our report, no significant difference of optimized linker length was observed, this might because that the length of cyclic shape plus alkyl chain linker could cover the distance for recognizing dimeric G-quadruplex.

To investigate the ability of **2-5** for inhibiting telomerase activity, instead of using general PCR coupling with gel shift TRAP assay, here we adopted an PCR-free telomerase assay using chronocoulometry coupled with hexaammineruthenium(III) chloride developed by our group, to conduct the evaluation[29,40]. The results (Fig. 4) indicated that, without adding G4 ligand, telomere DNA can be elongated by telomerase to $(TTAGGG)_{14.2}$, while with adding **5** (12.5 nM) to DNA (20 nM), telomere DNA elongation stopped at $(TTAGGG)_{13.2}$, continuously adding **5** to 25 nM, DNA elongation was stopped at $(TTAGGG)_{12.6}$, while, with adding 12.5 nM of **2**, telomere DNA could only be elongated to $(TTAGGG)_{9.0}$ (Fig. 4 and Fig. S17), and obtained shorter telomere DNA. This result confirmed that dimeric cNDI performed

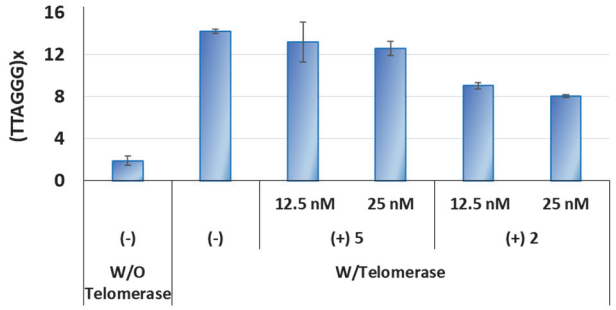

Figure 4. Telomere DNA elongation by telomerase was inhibited by **2** or **5**.

enhanced ability to inhibit telomerase ability than monomeric cNDI.

Conclusion

In this study, we designed and synthesized new cNDI derivatives, termed as cNDI-dimers for targeting dimeric G-quadruplex. cNDI-dimers exhibited higher preference for recognizing and stabilizing dimeric G-quadruplex structures than monomeric cNDI, and further possessed an enhanced ability to inhibit telomerase activity, which improved cNDI's potentiality as a promising telomerase inhibitor for further anti-cancer application.

Experimental section

Details list in the supplementary information.

Acknowledgements

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Key words: G-quadruplex • cyclic naphthalene diimide • dimer • stability

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Layout 1:

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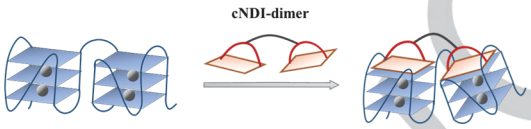

Ryusuke Takeuchi, [a] *Tingting Zou,*[ab] *Daiki Wakahara,*[a] *Yoshifumi Nakano,*[a] *Shinobu Sato,*[ab] *Shigeori Takenaka* [ab*]

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