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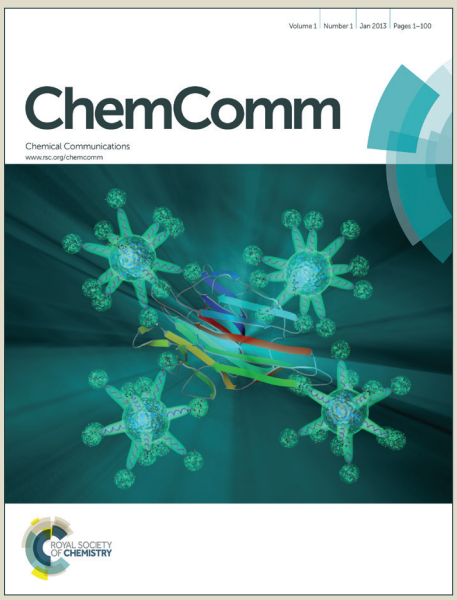

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Design of tetraplex specific ligands: cyclic naphthalene diimide

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Cyclic naphthalene diimide 1 bound to hybrid-type tetraplex DNA from 5'-AGGG(TTAGGG)$_3$-3' (K=8.6 × 10^6 M^{-1}) with 260-fold greater affinity than binding to double stranded oligonucleotide consisting of 5'-GGG AGG TTT CGC-3' and 3'-CCC TCC AAA GCG-5' (nK=3.3 × 10^4 M^{-1}) with 0.5 μM of IC$_{50}$ for telomerase activity.

Telomerase is a ribonucleoprotein that adds DNA sequence repeats to eukaryotic chromosome termini. Constitutive telomerase activity can lead to cell immortalization and cancer, so drugs that target the activity of this enzyme are garnering great interest. Telomere DNA is known to form tetraplex structures through guanine (G)-quartet formation,[1] and because this structure inhibits telomerase access, ligands that strongly bind these structures and stabilize these complexes are expected to be highly specific anticancer agents.[2,3] Until recently, these tetraplex-specific ligands have been exclusively designed to stack with the plane formed by G-quartet,[2] or to arrange their four substituents in the four grooves of tetraplex DNA.[3]

In this study, we designed and synthesized two cyclic naphthalene diimide compounds (**1** and **2**) by linking two substituents of different lengths with 2,2'-Cyclohexane-1,1-diyldiacetic acid. Human telomere DNA is known to have several tetraplex structure conformations, including hybrid, basket, chair, or propeller type.[4] All of these tetraplex structures retain spaces at both termini of three stacked G-quartets to permit stacking with ligand.[5] Cyclic naphthalene diimides as shown in Fig. 1A are expected to bind these sites through a stacking interaction (Fig. 1B). These compounds are also expected to have reduced binding for double stranded DNA (dsDNA) because of their linker substitutes (Fig. 1B&S1), so cyclic naphthalene diimides should have higher affinity for tetraplex DNA compared to dsDNA. This interaction has already been reported as classical intercalator.[6] Connecting substituents from the amido parts of naphthalene diimides might be more effective for reducing dsDNA affinity, due to blocking of one face in the intercalator plane with the aliphatic chain, so intercalation into duplex DNA be prevented, while end stacking onto G-tetrads can utilize a single face of the naphthalene diimide. To evaluate the selectivity of these derivatives for hybrid-type tetraplex DNA and dsDNA, we evaluated the interactions of **1** and **2** with 5'-AGGG(TTAGGG)$_3$-3' (a-core) and with double stranded oligonucleotide consisting of 5'-GGGAGGTTTCGC-3' and 3'-CCCTCCAAAGCG-5' (dsOligo). The a-core is known to form either hybrid or basket type tetraplexes in the presence of K^+ or Na^+, respectively,[5] so the presence of these ions was used to test selective binding to these conformations. The different conformations were confirmed by circular dichroism spectra of a-core in the presence of 0.10 M KCl or NaCl (Fig. S10A). The naphthalene diimide derivatives (**1**-**3**) had absorption maxima at 384 nm and showed hypochromic effects upon the addition of a-core or dsOligo. A hypochromic effect of 60% was observed for the interaction between non-cyclic naphthalene diimide **3** with dsOligo. A binding constant of 6.0 × 10^5 M^{-1} was obtained by Scatchard analysis of absorption change upon the addition of varied amounts of dsOligo (Table 1), which was in agreement with a similar report using calf thymus DNA.[7] The ratio of ligand per dsOligo used for binding was n=3, a reasonable result considering that a typical intercalator covers two base pairs upon binding to dsDNA, in addition to the expected relative difficulty of binding at terminal sites. The binding constant and binding number of ligand with DNA was estimated using the absorption change upon the addition of DNA, and was subsequently fitted with the Scatchard equation [$r/L=K(n-r)$; the DNA concentration used was defined as

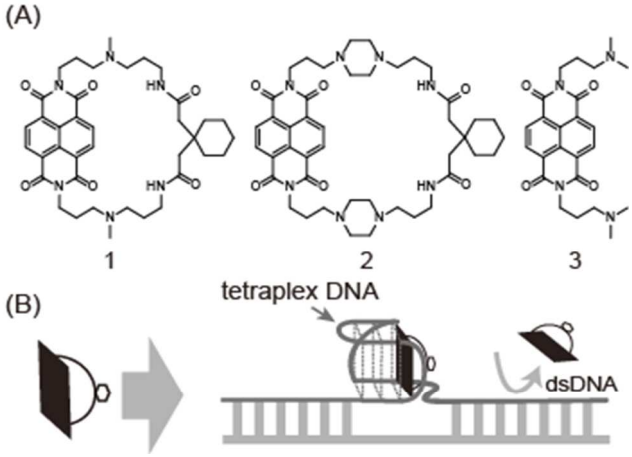

Figure 1. (A) Chemical structures of **1**-**3** and (B) concept of G-quartet specific ligand based on cyclic naphthalene diimide.

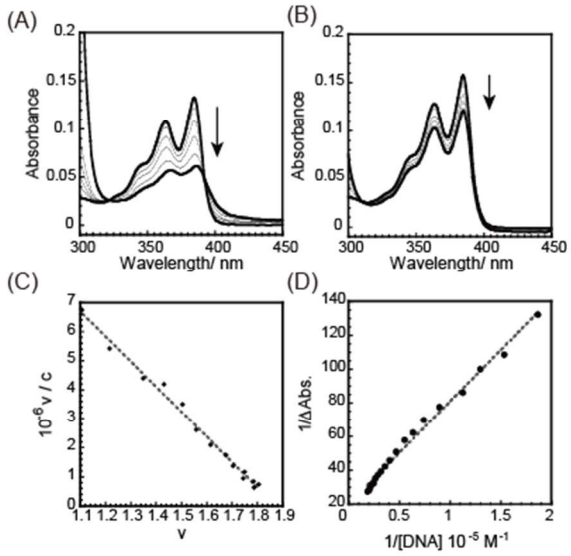

Figure 2. Spectrophotometric behaviours of 4.3 μM 1 in the presence of 0, 0.4, 0.8, 1.4, 2.2, and 9.5 μM a-core (A) or 5.1 μM 1 in the presence of 0, 6.5, 21, 34, 47, and 53 μM dsOligo (B) from top to bottom in 50 mM Tris-HCl (pH 7.4) and 100 mM KCl. Binding affinities of 1 with a-core or dsOligo were estimated by Scatchard (C) or Benesi-Hildebrand (D) plot, respectively.

per a-core or dsDNA molecule]. However, the product of the binding constant and binding stoichiometry (*nK*) was obtained using Benesi-Hildebrand analysis, with changing absorption using an excess amount of DNA. Fig. 2 shows binding the data of derivative **1** with a-core and dsOligo in the presence of KCl, and Table 1 shows the binding analysis of **1**-**3** with a-core or dsOligo in the presence of KCl or NaCl.

Naphthalene diimide **3** showed similar binding affinities with dsOligo in the presence of KCl and NaCl. The binding constant of **3** with a-core in the presence of K^+ was K=1.6 × 10^6 M^{-1} with a binding number of ca. 2. On the other hand, absorption of **3** showed a 20% hypochromic effect upon the addition of a-core in the presence of Na^+, and nK=1.1 × 10^5 M^{-1} by Benesi-Hildebrand for this interaction due to unsaturation even at high concentrations of a-core. Binding constants of **3** for a-core in K^+ are 16- or 3-times higher than a-core in Na^+ or dsOligo in K^+ and Na^+, respectively.

The absorption maximum of cyclic naphthalene diimide **2** at 384 nm showed a 50% hypochromic effect upon the addition of a-core in the presence of K^+, with a binding constant of K=1.5 × 10^6 M^{-1} and a binding amount of n=2 based on absorption changes using various amounts of a-core. The binding constant of **2** to a-core was similar to that of **3** in K^+ and Na^+, but then nK=3.0 × 10^4 M^{-1} obtained for **2**

Table 1 Binding parameters of **1** -**3** with a-core or dsOligo in the presence of K^+ or Na^+[a]

DNA	$10^{-5}K/M^{-1}$ (n)		
	1	2	3
a-core (K^+)	86 (1.9)	15 (1.6)	16 (1.5)
a-core (Na^+)	2.2*	1.1*	1.1*
dsOligo (K^+)	0.33*	0.30*	6.0 (2.8)
dsOligo (Na^+)	-	-	6.0 (3.0)

[a]50 mM Tris-HCl (pH 7.4) and 100 mM NaCl or KCl.

*Benesi-Hildebrand analysis, *nK*

Table 2 Stability of a-core or dsOligo by **1**-**3** in the presence of K^+ or Na^+[a]

DNA	T_m/°C	ΔT_m/°C		
		1	**2**	**3**
a-core (K^+)	68	3.0	2.0	6.0
a-core (Na^+)	56	1.3	1.0	1.0
dsOligo (K^+)	58	0	0	5.0
dsOligo (Na^+)	61	0	0	5.0

[a][ligand] : [DNA] = 1 : 1. 50 mM Tris-HCl (pH 7.4) and 100 mM NaCl or KCl.

Table 3 Thermodynamic data for the binding of **1**-**3** to a-core in the presence of K^+ or Na^+[a]

		1	**2**	**3**
K^+	$10^{-5}K/M^{-1}$	18.1	10.8	8.23
	n	1.9	1.9	2.1
	ΔH/kcal mol^{-1}	-13.4	-11.3	-5.87
	ΔS/cal mol^{-1}	-16.2	-10.4	7.38
Na^+	$10^{-5}K/M^{-1}$	3.27	0.59	3.77
	n	2.0	2.4	1.3
	ΔH/kcal mol^{-1}	-10.3	-63.4	-3.25
	ΔS/cal mol^{-1}	-9.17	-122.1	14.6

[a]50 mM potassium or sodium phosphate buffer (pH7.4)

and dsOligo revealed a 50-times higher preference of **2** for a-core than dsOligo. The introduction of a cyclic linker substitute in naphthalene diimide apparently retains the binding affinity for a-core while diminishing the affinity for ds DNA.

An unwinding experiment of plasmid DNA with Topoisomerase I using large amounts of **2** resulted in the unwinding of super helicity (Fig. S13B and S14D), suggesting that **2** can weakly bind dsDNA with partial stacking between base pairs in the DNA duplex.

We also synthesized a cyclic naphthalene diimide derivative with a shorter linker chain (**1**) in an attempt to reduce intercalation in dsDNA, then evaluated its interaction with a-core and dsOligo. Results show a binding constant of K=8.6 × 10^6 M^{-1} with n=2 for **1** with a-core, with a diminished affinity relative to **3** of nK=3.3 × 10^4 M^{-1} for dsOligo. Cyclic naphthalene diimide **1** did not show any unwinding of super coiled plasmid DNA, even using excess amounts of **1** in the Topoisomerase I assay (Fig. S13A and S14B&C). The data indicates that **1** has a 260-times preference for a-core over dsOligo. Although the binding affinity of **1** for a-core in the presence of Na^+ was similar to that of **2** and **3**, it is clear that **1** has a preference for hybrid tetraplex DNA compared to the basket conformation. This might be due to the fact that basket type a-core crosses its oligonucleotide chain over the G-quartet diagonally and disrupts access of **1** to the G-quartet plane.

Stabilization of DNA structure for **1**-**3** was tested using DNA melting temperature measurements (Fig. S11&S12), and results are summarized in Table 2. Similar stabilization effects for the use of **2** were observed in both a-core and dsOligo, which was expected due to the partial binding of this compound to dsDNA. Derivative **1** imparted little stabilization to dsOligo, highlighting the selective stabilization of hybrid-type a-core with **1**. Circular dichroism spectra of a-core showed little change upon the addition of **1** (Fig. S10), indicating that binding of **1** apparently does not disturb the structure of a-core. Isothermal titration calorimeter (ITC) measurements were also carried out for the binding of **1**-**3** with a-core. A typical experimental result is shown in Fig. S15 and results are summarized in Table 3. The order of binding affinities of **1**-**3** observed using ITC was in agreement with results obtained from changes in absorption spectra using varied concentrations of **1**-**3**. The non-cyclic **3** showed

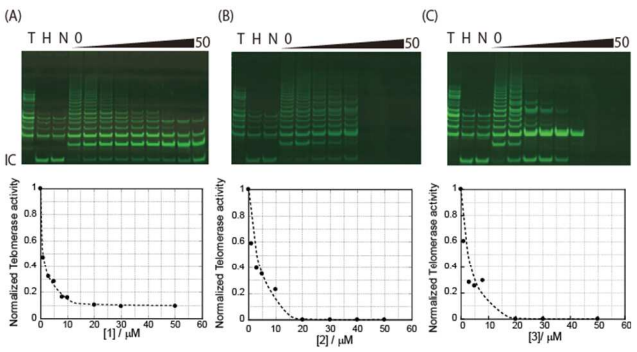

Figure 3. Telomerase inhibition by **1**-**3** in a TRAP assay. Increasing concentrations of **1** (A), **2** (B) or **3** (C) were added to the TRAP mixture in the presence of an internal control and analyzed by gel electrophoresis. TRAP activity was determined with 500 ng of an extract of a TRAPeze positive control cell line. IC_{50}s were determined as follows: ligand concentration under half telomerase activity with no ligand.

negative ΔH values with positive ΔS, which is in agreement with data previously reported.[8] Compounds **1** and **2** showed negative ΔS values suggesting an unfavourable process entropically, but the larger ΔH values apparently compensate for this disadvantage. Specific stabilization of **1** and a-core hybrid-type tetraplex derive from the larger ΔHs of this complex, which suggests effective stacking occurs between naphthalene diimide **1** and the G-quartet planes. ITC experiments of **1** and **2** for dsDNA couldn't estimate their binding affinities because of very small heat changes (Fig. S16).

To evaluate the abilities of these compounds to inhibit telomerase, the telomeric repeat amplification protocol (TRAP assay)[9] was carried out using various amounts of **1**-**3** (Fig. 3). All derivatives **1**-**3** displayed telomerase inhibition ability. The IC_{50}s of **1**, **2**, and **3** were 0.5, 3.0, and 3.0 μM, respectively, which were in agreement with previously observed binding affinities of these derivatives for a-core. Electropherograms of **2** and **3** show the disappearance of the lowest band as internal control for PCR in Fig. 3B and C, suggesting that **2** and **3** bind to dsDNA to inhibit PCR. On the other hand, **1** inhibited telomerase activity without any PCR inhibition (Fig. 3A). Interestingly, gel band, which is assigned as length to form one tetraplex structure ($(TTAGGG)_4$), remained even in the presence of high concentration of **1**. This behaviour was in agreement with electrochemical telomerase inhibition assay[10] (Fig. S17). This result suggests that TS-primer extends the length to form tetraplex structure and **1** bound to it and stabilized its structure to inhibit the telomerase reaction.

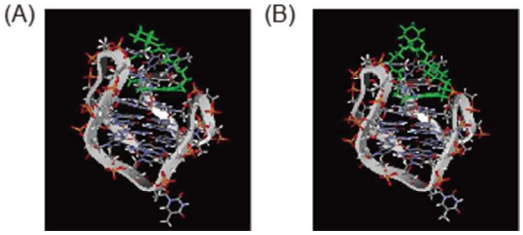

Figure 4. Computer modelling of the complex of **1** (A) and **2** (B) with a-core.

Figure 4 shows compute-modelled complexes consisting of **1** or **2** with a-core as a hybrid-type tetraplex structure. Both of the naphthalene diimide planes of **1** and **2** effectively stack with the a-core G-quartet one in these models, and the linker chain of **1** fits within the confines of the cavity created by the hybrid type tetraplex DNA. This model appears to show that the cyclohexane moiety of **1** is more effectively hidden within the hybrid type a-core cavity compared with **2**.

In this study, we successfully designed and synthesized ligands specific for hybrid type tetraplex DNA using cyclization of two linker chains of naphthalene diimide. Results suggest that optimization of the ring structure and/or length of linker chain might allow the development of highly specific telomere inhibitors that can work as highly effective anti-cancer agents.

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Notes and references

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† Electronic Supplementary Information (ESI) available: synthetic procedures of **1** and **2**, experimental procedures (Topoisomerase I assay, UV-Vis measurement, circular dichroism spectral measurement, melting curve measurement, AFM, ITC, electrochemical telomerase assay, and computer modelling), and Fig. S1-S17. See DOI: 10.1039/c000000x/

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