G-Quadruplexes

DOI: 10.1002/ange.201305366

Fluorescent-Ligand-Mediated Screening of G-Quadruplex Structures Using a DNA Microarray**

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Cell-cycle-synchronized DNA replication and cellular-environment-dependent gene expression are mainly controlled by proteins and DNAs with higher-order structures, such as G-quadruplexes (G4s). G4s are non-canonical nucleic acid structures that are formed by the stacking of planar G-quartets, and they are in equilibrium with random coils.[1] These structures were originally found in telomeric DNA at the ends of chromosomes.[2] The formation of telomeric G4s induces dysfunctions of telomeres in cancer cells.[3] Many G-quadruplex-forming oligonucleotides (GFOs) have been identified in promoter regions as well as in regions containing clusters of ribosomal RNA genes,[1,4] and they may be associated with transcriptional repression of oncogenes or inhibition of ribosome biogenesis in cancer cells, respectively.[4,5] Furthermore, G4s have been reported to be involved in replication,[6] DNA recombination,[7] and splicing processes.[8] Although relatively few GFOs have been identified thus far, bioinformatic studies suggest that a large number of GFOs are present in the genome.[9] These putative GFOs are concentrated in gene-regulatory elements, including gene promoters, nuclease hypersensitive sites, and cytosine—phosphate—guanosine (CpG) islands (CGIs), which are involved in epigenetic transcriptional modulation.[10] Therefore, G4s may be involved in regulatory mechanisms throughout the genome. Various direct or indirect methods for verifying G4 formation of DNA sequences have been reported,[11] but there has been no fast, high-throughput screening method for identifying large numbers of GFO candidates. Herein, we describe a method for a direct screening of GFOs that employs a fluorescent G4 ligand to probe a large microarray of 88737 probes in 16030 CGIs.

We have recently developed a series of macrocyclic G4 ligands that contain polyoxazole structures,[12] inspired by the natural G4 ligand telomestatin (**1**).[13] During our structural-development studies, one of the core structures, 7OTD,[12d] which has seven oxazole units in the macrocycle, was found to interact strongly and selectively with the known GFOs *c-myc*,[5a] *c-kit*,[14] *bcl-2*,[15] and *K-ras*,[16] without requiring a cationic moiety on the side chain. Based on these results, we developed the fluorescent G4 ligand L1BOD-7OTD, which consists of BODIPY linked to 7OTD.[12e] This ligand was effective for visualizing GFOs by a conventional electrophoretic mobility shift assay (EMSA) or fluorescent polarization (FP) titration.

We realized that a G4-selective fluorescent ligand might also be useful for screening GFOs on a DNA microarray. To evaluate this idea, we designed L1Cy5-7OTD (**3**), which consists of a Cy5 fluorophore linked to 7OTD (Figure 1), as a suitable ligand for high-throughput screening of GFOs in CGIs, which are major regulatory regions of gene expression and epigenetic transcription.[17] We considered that they would be suitable targets, because they contain many guanine-rich sequences, which are favorable for GFO formation.[10b]

L1Cy5-7OTD was synthesized by reacting L1H1-7OTD (**2**)[12d] with the *N*-hydroxysuccinimide (NHS) ester of Cy5 in the presence of sodium bicarbonate; then, its binding ability and selectivity for GFOs were examined. First, an assay on the displacement of thiazole orange[18] by **3** was carried out using five representative GFOs (telo24,[2,3] *c-myc*,[5a] *c-kit*,[14] *bcl-2*,[15] and *K-ras*)[16] and non-G4-forming double-stranded DNA (dsDNA). In this assay, **3** effectively displaced thiazole orange from all of the G4-forming DNA sequences, whereas no significant interaction was observed with dsDNA (Supporting Information, Table S1 and Figure S2). Subsequently, the binding ability and selectivity of **3** for GFOs were evaluated by EMSA. For EMSA, the same GFOs (telo24,[2,3] *c-myc*,[5a] *c-kit*,[14] *bcl-2*,[15] and *K-ras*)[16] were used, and dsDNA and poly(thymine) (poly-T) were evaluated as non-

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[**] We thank Dr. Hiroyuki Seimiya (JFCR. Japan) for helpful discussions. This work was supported in part by a Grant-in-Aid for Scientific Research (B) from JSPS (23310158), a Grant-in Aid for Challenging Exploratory Research from JSPS (21655060), a Grant-in-Aid for Scientific Research on Innovative Areas "Advanced Molecular Transformations by Organocatalysts" (23105013) from The Ministry of Education, Culture, Sports, Science and Technology (Japan), and a TUAT Next Gen. Grant 2013. K.N. is grateful for financial support from the Mukai Science and Technology Foundation, Tokyo (Japan), and the Mochida Memorial Foundation for Medical and Pharmaceutical Research, Tokyo (Japan). K.I., T.N., and M.T. are grateful for financial support in the form of JSPS Predoctoral Fellowships for Young Scientists.

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201305366.

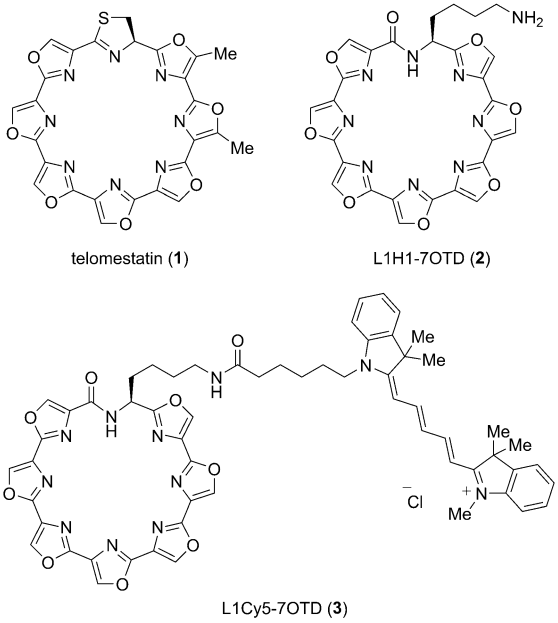

Figure 1. Chemical structures of telomestatin (**1**), L1H1-7OTD (**2**), and L1Cy5-7OTD (**3**). L1H1-7OTD is a telomestatin-mimicking macrocycle of polyoxazoles with a cationic side chain. The fluorescent probe L1Cy5-7OTD bears a Cy5 fluorophore on its side chain.

GFOs. These DNAs were subjected to electrophoresis on native 12% polyacrylamide gel in the presence or absence of **3**. The ligand bound to all the GFOs, but not to the DNA samples that do not form G4s (Table S2 and Figure S3). These two assays confirmed that the fluorescent G4 ligand **3** selectively interacts with GFOs, and should thus be suitable for the screening of GFOs in DNA microarray experiments.[19]

Therefore, we utilized **3** to probe a microarray of 88737 probes in 16030 mouse CGIs. The array was incubated with **3** in the presence of KCl (100 mM) for 1.5 h; then, unbound **3** was washed out with Tris buffer (Tris = tris(hydroxymethyl)-aminomethane). The fluorescence intensity at each probe site was measured, and 1998 probes (see the Supporting Information) were identified as candidate sequences for GFOs.[20]

To confirm the validity of this procedure, ten polynucleotides were randomly selected from the 1998 sequences (see the Supporting Information), and subjected to EMSA in the presence and absence of **3** to confirm the formation of a DNA–ligand complex. The DNA samples were stained with Stains-All, so that they were visualized as green bands (Figure 2). When DNAs interact with **3**, the resulting DNA–ligand complexes were visualized as red bands because of the fluorescence of **3** ($\lambda_{ex} = 648$ nm, 640–700 nm band-pass filter). All of the ten sequences yielded red bands, which confirms that these sequences interact with L1Cy5-7OTD and consequently indicates that they do indeed form G4 structures (Figure 2).[21]

To confirm the putative G4 structure formation of these ten sequences, we next performed CD spectroscopy in the presence of KCl (100 mM). Some spectra showed a characteristic positive Cotton effect at 265 nm or 290 nm, which

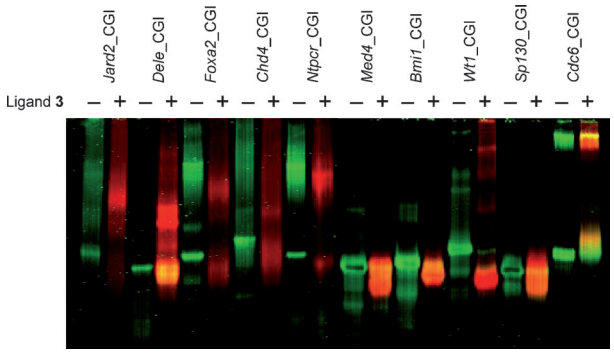

Figure 2. EMSA (12% native gel in 1×TBE buffer) results for ten sequences (10 μM) in the absence (−) and presence (+) of L1Cy5-7OTD (**3**; 100 μM). DNA was stained with Stains-All and detected using a 580–640 nm band-pass filter (green); therefore, DNA itself appears as green bands. DNA samples that had formed complexes with **3** were detected by making use of the fluorescence of **3** and employing a 640–700 nm band-pass filter (red); they are thus observed as red bands.

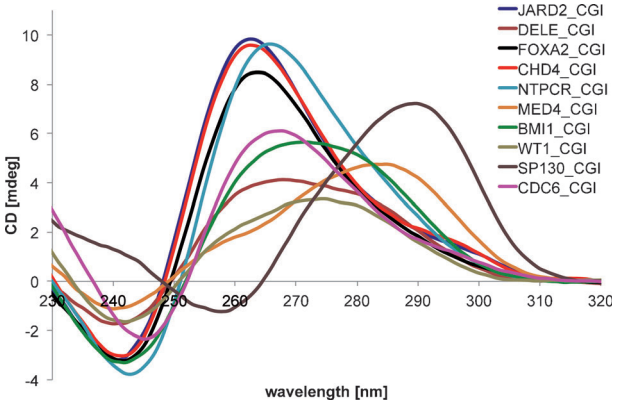

Figure 3. CD spectra in the presence of KCl (100 mM).

suggested that these DNAs form parallel-type G4 or anti-parallel-type G4 structures, respectively (Figure 3).[11e,22] We also performed thermal difference spectra (TDS) and isothermal difference spectra (IDS) analysis.[23] However, for some sequences, the formation of G4 structures could not be clearly detected with these analytical methods. Therefore, dimethyl sulfate (DMS) footprinting (10% DMS for 3 min in the absence or presence of K^+, as KCl)[11c] was then carried out to confirm the formation of G4 structures by the ten sequences.

Representative results for the *Sp130* and *Dele* sequences are shown in Figure 4 (see also Figure S7). Four sets of guanine dinucleotides (2–3, 5–6, 7–8, 10–11 in *Sp130*) and four sets of guanine trinucleotides (4–6, 7–9, 14–16, 17–19 in *Dele*) were protected against a methylation reaction with DMS (as the methylating reagent; Figure 4), which is consistent with the idea that these guanines contribute to the construction of G4 structures through the formation of G-quartet planes. The other sequences were also confirmed to form G4 structures in a similar manner (Figure S7).[24–26]

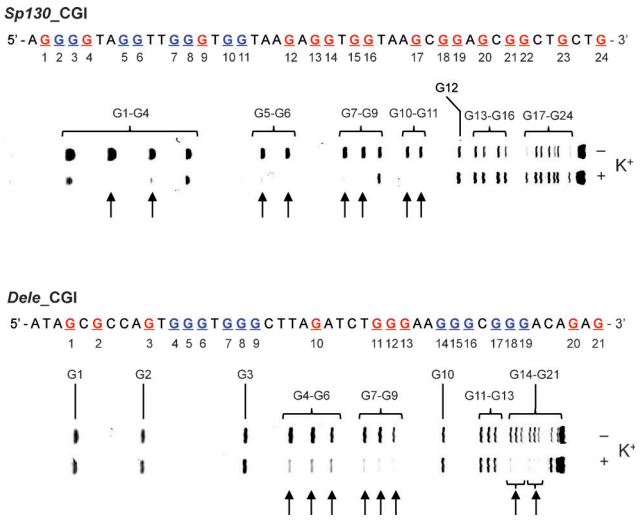

Figure 4. DMS footprinting analysis of two representative GFO candidates (*Sp130*_CGI and *Dele*_CGI) in the absence (−) and presence (+) of KCl (100 mM). The guanines that were protected against a methylation reaction are indicated with arrows and shown in blue, while guanines that were not protected are shown in red. These samples were run on a denatured polyacrylamide gel (20%; 7 M urea) with 1×TBE buffer.

To examine the possible roles of the newly identified GFOs, we performed gene ontology (GO) analysis.[27,28] We searched for genes located proximal to the GFOs, and identified 1578 genes (see the Supporting Information). GO analysis in the biological process category (chemical reactions of a living organism) indicated that these genes were mainly associated with metabolic processes, transcriptional regulation, biosynthetic processes, and developmental processes (see the Supporting Information). Thus, the newly defined GFOs may have regulatory roles in these categories.

In summary, we have developed a fast, high-throughput method for screening GFOs by using the fluorescent G4 ligand L1Cy5-7OTD (**3**) to probe a microarray of 88737 probes in 16030 mouse CGIs. Among them, 1998 sequences were discovered as candidate GFOs. Ten randomly selected candidates were confirmed to form G4 structures in the presence of potassium cations by means of CD spectroscopy and DMS footprinting. Gene ontology analysis of genes proximal to these GFOs showed that they were mainly associated with metabolic processes, transcriptional regulation, biosynthetic processes, and developmental processes, which suggests that these GFOs may have regulatory functions in these categories.

Experimental Section

Microarray analysis: A solution of L1Cy5–7OTD (10 nM) in binding buffer (50 mM Tris-HCl, 100 mM KCl, pH 8.0) with 1×Hi-RPM Hybridization Buffer (Agilent) was applied to a Mouse CpG Island Microarray (Agilent) and incubated for 1.5 h at 20 rpm at 25 °C. The microarray was washed in Tris-HCl buffer for 5 min at 25 °C, and scanned with an Agilent DNA Microarray scanner using Surescan high-resolution technology (Agilent). Signals were extracted from the scanned microarray image with Agilent Feature Extraction Software to identify DNA probes bound to L1Cy5-7OTD on the microarray. Triplicate analysis was carried out, and averaged values of processed signals were calculated. Microarray data analysis was carried out with Microsoft Excel 2010.

CD spectra: CD spectra were recorded on a J-720 spectropolarimeter (JASCO, Tokyo, Japan) using a quartz cell (Agilent, microcell: 50 μL, 10 mm) and an instrument scanning speed of 500 nm min^{-1} with a response time of 1 s, over a wavelength range of 220–320 nm. The purified nucleotides (Operon Biotechnology, Tokyo, Japan) were dissolved as stock solutions (50 μM) in TE buffer to be used without further purification. These nucleotide solutions were diluted to 1.0 μM (50 μL) with Tris-HCl (50 mM) at pH 7.5 and with KCl buffer (100 mM). Subsequently, these solution were annealed by heating at 96 °C for 2 min, and then slowly cooled to room temperature. Finally, CD spectra are the average of ten scans taken at 25 °C.

Dimethyl sulfate (DMS) footprinting: DNA samples without K^+ and with K^+ were prepared as follows: In the absence of K^+, 5′-end-FITC-labeled DNAs (100 μM, 5.0 μL) were diluted with MilliQ (84 μL) and Tris buffer (pH 7.5, 500 mM, 11 μL). In the presence of K^+, 5′-end-FITC-labeled DNAs (100 μM, 5.0 μL) were diluted with MilliQ (84 μL) and Tris buffer (500 mM)–KCl (1M; pH 7.5, 11 μL). These samples were heated to 95 °C for 3 min and then cooled to room temperature. The samples were treated with dimethyl sulfate (DMS) in ethanol (10%, 10 μL) for 3 min to methylate the DNA. The reactions were stopped by the addition of DMS stop solution (10 μL), which consists of sodium acetate buffer (3M, pH 7.0; Sigma Aldrich)/2-mercaptoethanol (Sigma Aldrich)/tRNA (100 mg mL^{-1}, Roche Diagnostics)/MilliQ (25:21:1:9, *v*/*v*/*v*/*v*). Ethanol (300 μL) was added to the resulting solutions at −80 °C, and these reaction mixtures were incubated for 30 min at −80 °C. Then, they were centrifuged at 15000 rpm for 30 min at 4 °C, and the supernatant fluids were removed. Sodium acetate buffer (3M, 10 μL), MilliQ (100 μL), and ethanol (250 μL) were added to the DNA pellets, and centrifugation was repeated under the same conditions as before. The resulting supernatant fluids were removed to obtain the DNA pellets. Ethanol in MilliQ (70%, 800 μL) was added to each sample. After centrifugation at 15000 rpm for 5 min at 4 °C, the resulting supernatant fluids were removed to leave the DNA pellets (this operation was repeated twice). The resulting DNA pellets were dried in air, incubated with piperidine solution in MilliQ (10%, 100 μL) for 30 min at 95 °C, and then freeze-dried. The DNA pellets were taken up in loading buffer (5.0 μL), which consists of formamide/EDTA (0.5M, pH 8.0)/MilliQ (197:10:43, *v*/*v*/*v*), and the resulting samples were heated at 95 °C for 3 min, then cooled rapidly to 4 °C. Finally, aliquots (1.0 μL) were loaded on a 20% polyacrylamide gel containing urea (7M), which were developed at 1000 V for 10 min, then at 2500 V for 95 min in 1×TBE (TBE = Tris–Borate–EDTA) buffer. The gels were scanned with a phosphorimager (Typhoon 8600, GE Healthcare) using a 526 nm short-pass filter.

Received: June 21, 2013
Revised: September 10, 2013
Published online: October 21, 2013

Keywords: DNA microarrays · fluorescence · G-quadruplexes · high-throughput screening · oligonucleotides

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[19] The differences in the fluorescence response of **3** in the presence of G4-forming oligonucleotides were small (Figure S4).
[20] Bioinformatic studies indicate that the ($G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}N_{1-7}G_{3+}$) sequence, which involves four sets of guanine trinucleotides, is a candidate G4-forming motif (G = guanine, N = any nucleotide).[9] However, some G4-forming sequences, such as thrombin aptamer,[29] *c-Kit87up*,[14] *c-Myb*,[30] *Rb*,[31] and *c-myc23456*,[32] do not possess four sets of guanine trinucleotides. Considering this fact, we selected sequences as candidate GFOs on the basis of the following criteria: 1) the fluorescence intensity value of the probe is more than 1704, which is five times the average signal value of 341;[33] and 2) the probe contains at least four sets of guanine dinucleotides ($G_{2+}N_{1+}G_{2+}N_{1+}G_{2+}N_{1+}G_{2+}$). Among the 1998 probes that were identified using these criteria, 296 possess guanine trinucleotides in ($G_{3+}N_{1+}G_{3+}N_{1+}G_{3+}N_{1+}G_{3+}$) sequences, and the others have dinucleotides in ($G_{2+}N_{1+}G_{2+}N_{1+}G_{2+}N_{1+}G_{2+}$) sequences.
[21] Several oligonucleotides showed multiple bands, probably because of their multimeric and/or multiple structures. We thank the referees for helpful discussions.
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[23] We also carried out thermal difference spectra (TDS) and isothermal difference spectra (IDS); see Figures S5 and S6.
[24] We found seven GFOs with 2 tetrads, namely *Jard2*_CGI, *Foxa2*_CGI, *Chd4*_CGI, *Bmi1*_CGI, *Wt1*_CGI, *Sp130*_CGI, and *Cdc6*_CGI, in the ten sequences that we examined.
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