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An exonuclease I hydrolysis assay for evaluating G-quadruplex stabilization by small molecules

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ABSTRACT

Telomere length homeostasis is a prerequisite for the generation and growth of cancer. In >85% cancer cells, telomere length is maintained by telomerase that add telomere repeats to the end of telomere DNA. Because the G-rich strand of telomere DNA can fold into G-quadruplex that inhibits telomerase activity, stabilizing telomere quadruplex by small molecules is emerging as a potential therapeutic strategy against cancer. In these applications, the specificity of small molecules toward quadruplex over other forms of DNA is an important property to ensure no processes other than telomere elongation are interrupted. The evaluating assays currently available more or less have difficulty identifying or distinguishing quadruplex-irrelevant effect from quadruplex stabilization. Here, we describe an exonuclease I hydrolysis assay that evaluates quadruplex stabilization by DNA-interacting compounds, discriminates inhibitory effect from different sources and helps determine the optimal compound concentration.

INTRODUCTION

Chromosomes in human cells are capped at both ends with (TTAGGG)*n* DNA arrays called telomere that is essential in maintaining chromosomal integrity and cellular viability. Due to the end-replication problem, telomere shortens during each round of DNA replication and this shortening, if continued without compensation, eventually limits the division potential of cells. Telomere length homeostasis is essential for the generation and growth of cancer. In >85% cancer cells, telomere shortening is compensated by telomerase, a ribonucleoprotein reverse transcriptase that adds telomeric repeats to telomere ends (1). Telomere DNA stretches for several thousand base pairs in double-stranded form and terminates with a single-stranded G-rich overhang that serves as a substrate for telomerase. The G-rich overhang can fold in the presence of K^+ or Na^+ into a four-stranded G-quadruplex (2) that is not a substrate for telomerase (3,4). Stabilization of quadruplex by small molecules has been shown to inhibit telomerase activity (5) and induce growth arrest, senescence and apoptosis in cancer cells (6). For this reason, there is currently intense interest in exploring quadruplex-stabilizing compounds to inhibit telomere maintenance by telomerase as a potential therapeutic strategy against cancer (7).

Except the telomere overhang, the DNA in a chromosome is double stranded and protected by proteins. However, it opens in many crucial biological processes, such as replication, transcription and promoter recognition, in which the DNA is present in single-stranded form. For quadruplex targeting, the structural specificity toward quadruplex is a key property determining the therapeutic potency and toxicity of small molecules. An ideal compound should be such that it specifically stabilizes quadruplex structure, but does not interact with DNA in the single-stranded form to avoid interfering with other biological processes. At present, several methods are available for evaluating the properties of quadruplex-stabilizing compounds. Selectivity toward quadruplex over other structures can be analyzed by the dialysis assay (8) and biosensors (9–12). Stabilization of quadruplex can be examined by the melting assay (13), the DNA polymerase stop assay (14), the PCR stop assay (15) and the telomerase repeat amplification protocol (TRAP) (16). The methods for analyzing quadruplex stabilization, except the melting assay, involve both single-stranded and quadruplex DNA. Quadruplex can also open to become unstructured during analysis. Therefore, such methods may experience cross-interference from

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quadruplex-irrelevant interactions. The lack of independent assessment for the effect on different structures makes them difficult to distinguish quadruplex-specific effect from quadruplex-irrelevant ones.

In the present work, we describe an exonuclease I assay for evaluating quadruplex stabilization by small molecules. The assay uses a quadruplex-forming and a non-quadruplex-forming oligomer as substrates (Figure 1). The formation of quadruplex in the former oligomer inhibits its hydrolysis and quadruplex stabilization enhances the inhibition. By comparing independent hydrolysis of the two substrates, the assay can evaluate quadruplex stabilization, discriminates inhibitory effect from different sources and helps determine the optimal compound concentration.

MATERIALS AND METHODS

Oligonucleotides, compounds and enzyme

Oligonucleotides were purchased from Sangon Technology (Shanghai, China). Fluorescent $(G_3T_2A)_3G_3$ labeled at the 5′ end with a fluorescein (FAM) and the 3′ end a tetramethylrhodamine (TMR) was purchased from TaKaRa Biotech (Dalian, China). 5,10,15,20-Tetra(*N*-methyl-4-pyridyl)porphine ($TMPyP_4$) and 3,3′-diethyloxadicarbocyanine iodide (DODC) were from Sigma. 3,6-bis(1-methyl-4-vinylpyridinium)carbazole diiodide (BMVC) was a generous gift from Dr T.C. Chang at the Institute of Atomic and Molecular Sciences, Academia Sinica, Taipei, Taiwan, ROC. Exonuclease I from *Escherichia coli* was purchased from TaKaRa Biotechnology.

Native gel electrophoresis of ^{32}P-labeled oligonucleotides

Oligonucleotides were labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase (Fermentas, Lithuania) and dissolved in 1× TBE buffer containing 150 mM KCl. The samples were heated at 95°C for 5 min and slowly cooled down to room temperature. After incubation at 37°C for 30 min in the absence or presence of compounds, the samples were resolved on 12 or 19% polyacrylamide gel containing 150 mM KCl. Autoradiograph was obtained by exposing to X-ray film.

Fluorescence resonance energy transfer (FRET)

Measurements were carried out on a Spex Fluorolog-3 spectrofluorometer (HORIBA Jobin Yvon, France) at 25°C at various concentrations of K^+ as described (17) except that the extent of FRET was calculated as $I_D/(I_A + I_D)$, where I_D and I_A are the fluorescence intensities of the donor (FAM) and acceptor (TMR), respectively.

Exonuclease I hydrolysis assay

Oligonucleotides were labeled at the 5′ end with [γ-^{32}P]ATP using T4 polynucleotide kinase (Fermentas, Lithuania). Hydrolysis was carried out in 15 µl reaction buffer containing 67 mM Tris–HCl, pH 7.4, 6.7 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mg/ml BSA, 5 nM oligonucleotide and indicated salt (18). Before the addition of compound and Exonuclease I, samples were heated at 95°C for 5 min, slowly cooled down to 37°C and maintained for 20 min. Hydrolysis was initiated by addition of Exonuclease I and maintained at 37°C. Reactions were stopped by adding 15 µl stop solution containing 10 mM EDTA, 10 mM NaOH and 0.1% bromphenol blue in formamide solution. Samples were electrophoresed on 19% denaturing polyacrylamide gel containing 7 M urea for 20 min, autoradiographed on a Typhoon phosphor imager (Amersham Biosciences, Uppsala, Sweden) and quantified with the software ImageQuant 5.2.

UV-melting analysis

Here, ~2 µM $(G_3T_2A)_3G_3$ was prepared in 10 mM potassium phosphate buffer (pH 7.4), 1 mM EDTA, with the final K^+ concentration adjusted to 150 mM. The samples were heated at 95°C for 5 min and slowly cooled

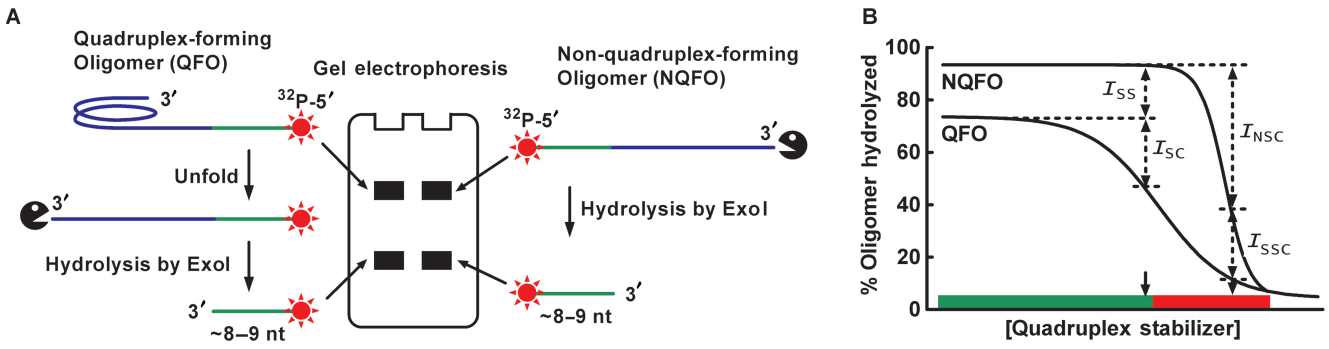

Figure 1. Schematic illustration of analysis of quadruplex stabilization by exonuclease I hydrolysis. (**A**) G-quadruplex-dependent inhibition of hydrolysis by exonuclease I. The assay uses a quadruplex-forming and non-quadruplex-forming oligomer (QFO and NQFO) labeled with ^{32}P at the 5′ end. The quadruplex at the 3′ end of the QFO cannot be processed by exonuclease I until it becomes unfolded. The hydrolysis does not proceed to the very end producing a short fragment of ~8–9 nt which is separated from the input oligonucleotide by gel electrophoresis based on their size and visualized by radioautography. (**B**) Information provided by the exonuclease I hydrolysis assay. The assay generates hydrolysis curve for the two oligomers and clarifies inhibition from different sources: I_{SS}, structure-dependent inhibition by salt in the medium; I_{SC}, structure-dependent inhibition by compound; I_{SSC}, structure-dependent inhibition by salt and compound; I_{NSC}, non-specific inhibition by compound at high concentration via interaction with DNA or/and protein. Green bar indicates the concentration range within which the compound stabilizes quadruplex without affecting single-stranded substrate; red bar indicates the concentration range within which the compound affects hydrolysis of single-stranded substrate.

down to room temperature. After incubation at 37°C for 30 min in the absence or presence of 2 μM compounds, denaturations were carried out as described (19) on a Beckman DU-640 UV–Vis spectrophotometer equipped with a digital circulating water bath.

Telomerase activity assay

Telomerase activity was analyzed by the TRAP method using extracts from exponentially growing HeLa cells as described (20). Primer extension was carried out in the presence of an internal standard (IS) and various concentrations of compounds. PCR products were resolved on 12% polyacrylamide gel, stained with ethidium bromide, recorded and quantitated on a ChemiImager 5500 (Alpha Innotech, San Leandro, CA, USA). Telomerase activity was expressed as percent of the control in which no compound was added using the formula $(TP/TP_0) \times (IS_0/IS) \times 100$, where TP_0 and IS_0 are the integrated density of telomerase products and IS of the control, respectively; TP and IS the corresponding integrated density obtained in the presence of compound.

RESULTS

Our method used Exonuclease I to evaluate the stabilization of quadruplex by small molecules. This enzyme successively hydrolyzes nucleotides from the 3′ end of single-stranded, but not double-stranded DNA (18). Two ^{32}P-5′-end-labeled oligonucleotides, $T_{24}(G_3T_2A)_3G_3$ and T_{24}GTGTGAGTGGAGGTGTGAGGT denoted as T24G21 and T24RG21, respectively, were used as substrates. The T24G21 carried a core sequence of the G-rich strand of human telomere DNA at the 3′ end of the T_{24} and the T24RG21 was composition and length matched to the T24G21, but with the core sequence randomized to abolish quadruplex formation. As the most extensively studied sequence, the G-rich strand of human telomere DNA forms mixed parallel–antiparallel-stranded quadruplex in K^+ solution (21–23). The $(G_3T_2A)_3G_3$ moiety in T24G21 formed intramolecular quadruplex in 150 mM K^+ solution as demonstrated by a faster migration relative to that of T24RG21 in native gel electrophoresis (Figure 2A). Figure 2B shows the K^+ concentration dependence of quadruplex formation by $(G_3T_2A)_3G_3$ detected by fluorescence resonance energy transfer (FRET) analysis. The $(G_3T_2A)_3G_3$ was labeled at the 5′ end with FAM as a donor and the 3′ end with TMR as an acceptor. The formation of intramolecular quadruplex brought the 5′ and 3′ ends to close proximity allowing FRET between the two fluorophores to occur (17). To examine if the Exonuclease I can distinguish quadruplex structure, hydrolysis was carried out at various concentrations of K^+. Exonuclease I is highly processive, but the hydrolysis does not proceed to the very 5′ end of a DNA leaving a short fragment of ~8–9 residues (18). The hydrolysis product and the original input substrate remaining were easily separated by gel electrophoresis and visualized as two distinct bands on

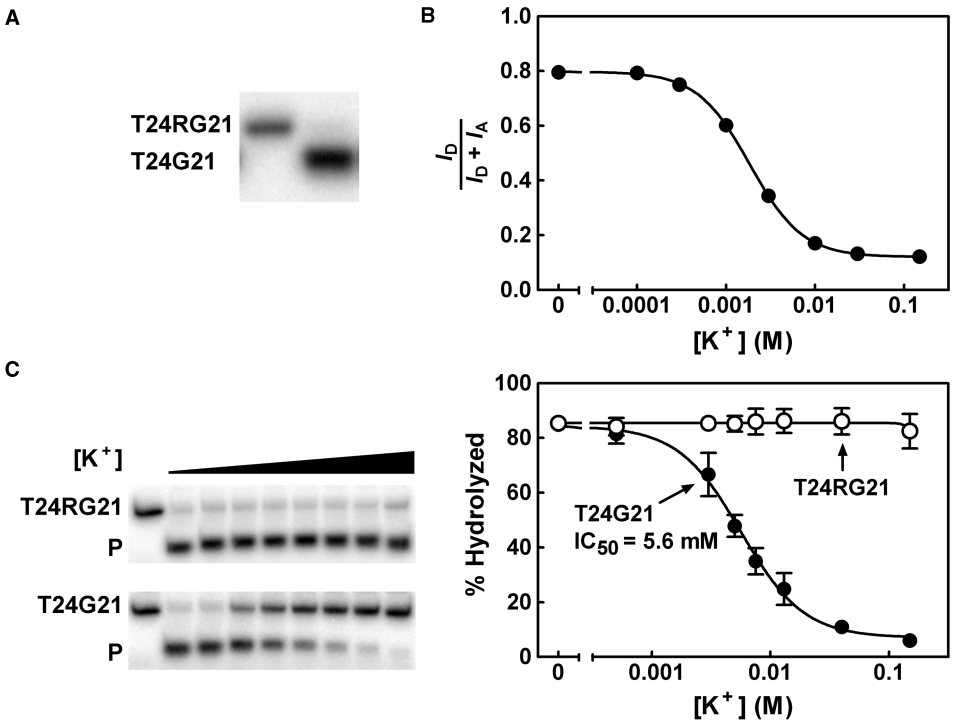

Figure 2. Quadruplex formation by T24G21 and its resistance to hydrolysis by exonuclease I. (**A**) Native gel (19%) electrophoresis showing quadruplex formation by T24G21 in 150 mM K^+. (**B**) Quadruplex formation by $(G_3T_2A)_3G_3$ as a function of K^+ concentration examined by fluorescence resonance energy transfer (FRET). (**C**) Hydrolysis of T24RG21 (open circles) and T24G21 (filled circles) by exonuclease I as a function of K^+ concentration. (Left) Separation of input oligonucleotide and hydrolysis product (P) by gel electrophoresis. Lane 1: no exonuclease, lanes 2–9: treated with 0.04 U exonuclease I for 20 min in buffer containing increasing concentrations of K^+. LiCl was added to make the total concentration of monovalent cation to 150 mM. (Right) Quantification of oligonucleotide hydrolysis. Data represent the mean of three experiments with standard deviation.

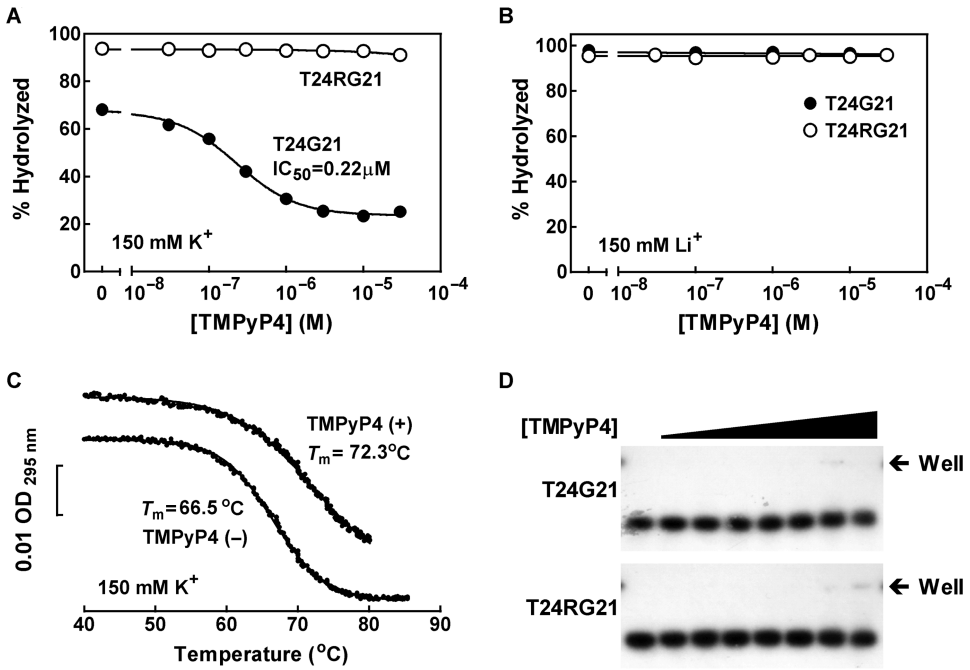

Figure 3. Effect of TMPyP4 on oligonucleotides. (**A** and **B**) Hydrolysis of T24G21 (filled circles) and T24RG21 (open circles) by exonuclease I (3 U) for 1 h in the presence of 150 mM of (A) K^+ or (B) Li^+. (**C**) Thermal stability of $(G_3T_2A)_3G_3$ quadruplex in the absence and presence of equimolar TMPyP4. Curves were nudged along vertical axis to avoid overlap. (**D**) Electrophoresis behavior of T24G21 and T24RG21 in 12% native gel. Data in (A) represent the mean of two hydrolysis experiments with range. Samples in (D) were prepared as in (A) but without exonuclease hydrolysis.

radioautograph (Figure 2C, left). In the absence of K^+, both oligonucleotides were effectively cleaved. Additions of K^+ resulted in resistance to hydrolysis of T24G21 in a concentration-dependent manner (Figure 2C, right) similar to that of the quadruplex formation revealed by FRET. The K^+-induced resistance to hydrolysis is explained by quadruplex formation of the T24G21, but not by a direct effect of K^+ because the hydrolysis of the T24RG21 that does not form quadruplex was not affected.

The above results show that the method is able to assess quadruplex stabilization by resistance to hydrolysis. We then carried out assays with three chemical compounds, TMPyP4, BMVC and DODC at physiological concentration (150 mM) of K^+ (Figures 3–5). TMPyP4 is a cationic porphyrin compound, which has been well characterized with respect to its effect on quadruplex. It stabilizes human telomere quadruplex (24–27), has good selectivity for quadruplex over single-stranded and duplex DNA (10) and inhibits telomerase activity (28). In line with this, the TMPyP4 was found to inhibit the hydrolysis of T24G21 by exonuclease I in a concentration-dependent manner with an IC_{50} at 0.22 μM (Figure 3A). This inhibition was quadruplex-dependent because the hydrolysis of the unstructured T24RG21 was not affected. Since quadruplex does not form in Li^+ solution (2), replacing K^+ with Li^+ in the assay removed the resistance of T24G21 to hydrolysis (Figure 3B), further supporting that the inhibition was dependent on quadruplex. The inhibition could be explained by quadruplex-stabilization by TMPyP4, which, at 1:1 molar ratio to DNA, increased the melting temperature (T_m) of $(G_3T_2A)_3G_3$ quadruplex by 6.7°C (Figure 3C). The interaction of TMPyP4 with the two oligonucleotides was examined by native gel electrophoresis (Figure 3D). No obvious effect on the electrophoresis behavior was observed.

The BMVC is a carbazole derivative that has been shown to stabilize human telomere quadruplex and inhibit telomerase activity (29). In our assay, BMVC inhibited the hydrolysis of T24G21 in a concentration-dependent manner with an IC_{50} at 0.42 μM (Figure 4A). Unlike TMPyP4, BMVC also inhibited the hydrolysis of T24RG21 with an IC_{50} at 2.43 μM indicating that quadruplex-irrelevant inhibition was present. This was further supported by the fact that when K^+ was substituted with Li^+ to abolish the formation of quadruplex, both substrates were similarly hydrolyzed (Figure 4B). BMVC also increased the T_m of $(G_3T_2A)_3G_3$ quadruplex (Figure 4C) (29). This quadruplex stabilization explains the different inhibition between T24G21 and T24RG21. In the native gel electrophoresis with K^+ (Figure 4D), the retaining of oligonucleotides in the wells at high BMVC concentrations suggests that BMVC-induced aggregation in both substrates. Similar results were also obtained in electrophoresis with Li^+ (data not shown). These results provide an explanation for the quadruplex-irrelevant inhibition because the concentrations at which aggregates appeared (Figure 4D) correlated with the concentrations at which inhibition occurred in the assays containing Li^+ (Figure 4B).

The DODC, a carbocyanine dye, has been reported to bind dimeric hairpin quadruplexes (30), but its effect on telomerase has been inconsistent. It has been shown to reduce telomerase activity in pheochromocytoma PC-12

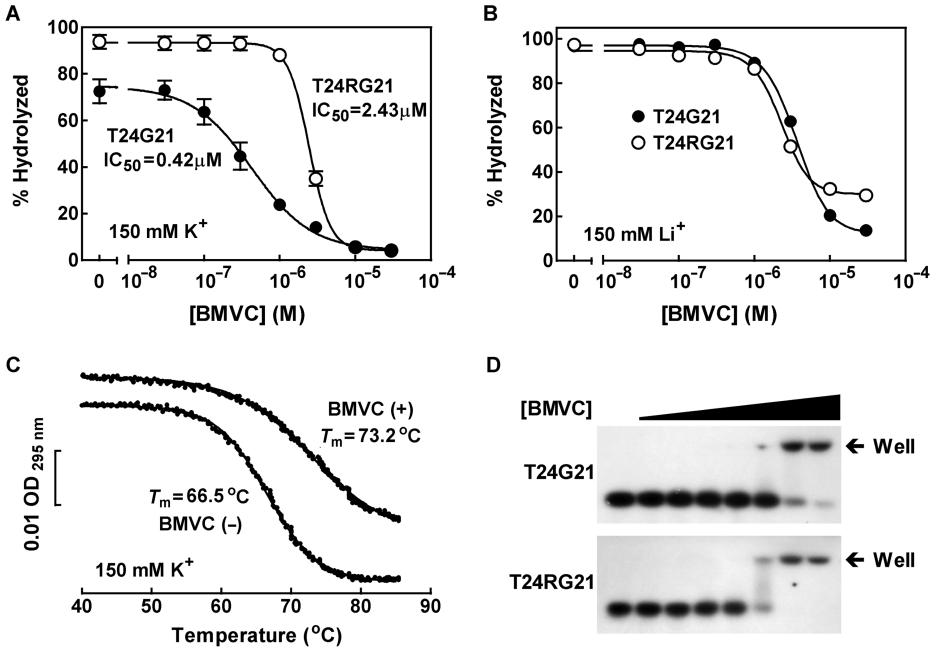

Figure 4. Effect of BMVC on oligonucleotides. Experiments were carried out as in Figure 3 except that BMVC was used instead of TMPyP4.

cells in a concentration-dependent manner after 12 h treatment (10–100 μM) (31). In another study, DODC was found to inhibit telomerase in Nasopharyngeal Carcinoma NPC-Tax, but not in NPC-TW01 cells, when assayed with lysate from cells treated with 0.7 μM of DODC for 1–3 days. When DODC was added directly to the assay medium, inhibition was observed at 10 μM for NPC-TW01 and 500 μM for NPC-TW01 cells (32). In our study, DODC at the concentrations tested had little effect on the hydrolysis of the two substrates in K^+ (Figure 5A) or Li^+ (Figure 5B) solution, the stability of T24G21 quadruplex (Figure 5C) and the electrophoresis behavior of both T24G21 and T24RG21 (Figure 5D).

The compounds were further assayed for their effect on telomerase activity by the TRAP method. The telomere substrate was extended by telomerase in the presence of increasing concentrations of compounds and extension products were amplified by PCR in the presence of an IS (20). TMPyP4 inhibited telomerase activity in a concentration-dependent manner (Figure 6A), but quantitation of inhibition at high concentrations was difficult because TMPyP4 also inhibited the PCR as was demonstrated by the decrease in the intensity of the IS band with increase in compound concentration. BMVC also showed concentration-dependent inhibition on telomerase activity with an IC_{50} (Figure 6B) at the same order of magnitude as the one derived from the exonuclease I hydrolysis of T24G21 in K^+ solution (Figure 4A). This inhibition, as we can see from Figure 4, was likely a combination of both quadruplex stabilization and quadruplex-irrelevant contribution. DODC, which did not inhibit the exonuclease hydrolysis (Figure 5), did not inhibit telomerase either (Figure 6C).

Except for the telomere DNA, quadruplex-forming sequences have been found in many other essential regions of chromosomes, for example, the promoter of BCL-2, retinoblastoma gene, hypoxia-inducible factor 1α, *c-myc* oncogene (33). Bioinformatic analysis has revealed several hundred thousand putative quadruplex sequences in human genome (34,35). It is believed that quadruplex is involved in certain biological processes such as regulation of gene transcription and telomere elongation. Interest is growing in recent years in searching for quadruplex-stabilizing compounds for therapeutic purposes (36). In principle, our exonuclease hydrolysis assay provides a general method for evaluating quadruplex-stabilization of any other sequences by using appropriate target sequence. Figure 7 shows such an example in which the quadruplex of the *c-myc* gene promoter sequence (GAGGGTGGGGAGGGTGGGG AAG) was used. Since the *c-myc* quadruplex is much more stable than the human telomere one [85 (37) versus 65°C (38) in melting temperature in 100 mM K^+], the assays had to be performed in solution containing reduced concentration of K^+ to detect the effect of small molecules. Similar to their effect on human telomere quadruplex, both TMPyP4 and BMVC stabilized *c-myc* quadruplex, but with much lower IC_{50}. DODC did not affect *c-myc* quadruplex. The lower IC_{50} of TMPyP4 for *c-myc* quadruplex than for human telomere quadruplex is in agreement with a previous report that the former structure was about seven times more competitive than the latter in binding to TMPyP4 (10). The different values of IC_{50} of TMPyP4 and BMVC for human telomere and *c-myc* quadruplexes demonstrate that the exonuclease assay can discriminate their effect on different structures under different conditions.

DISCUSSION

Several methods are currently available for analyzing quadruplex stabilization by small molecules. In the widely

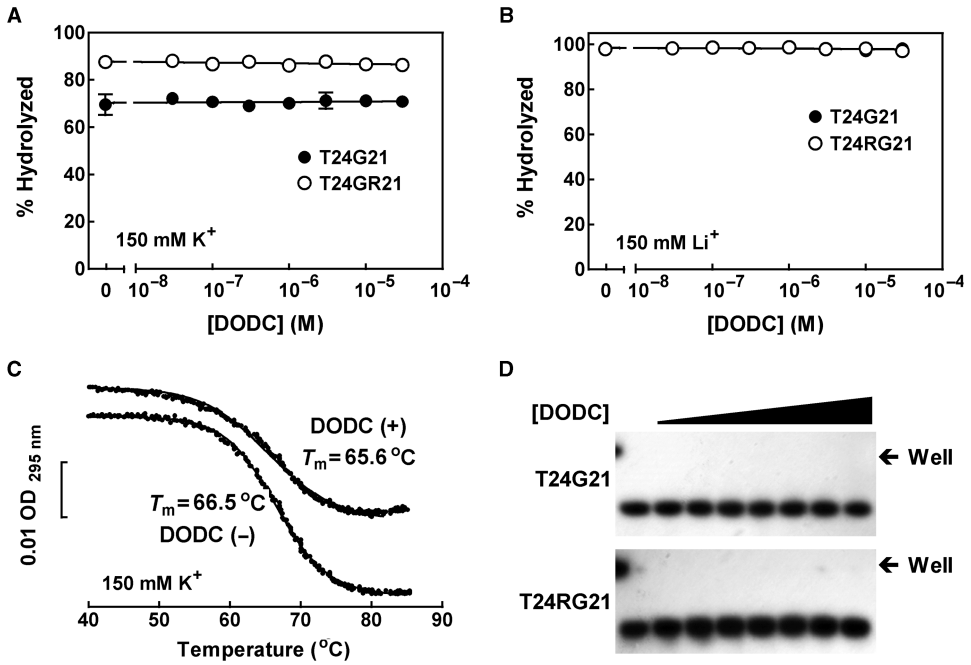

Figure 5. Effect of DODC on oligonucleotides. Experiments were carried out as in Figure 3 except that DODC was used instead of TMPyP4.

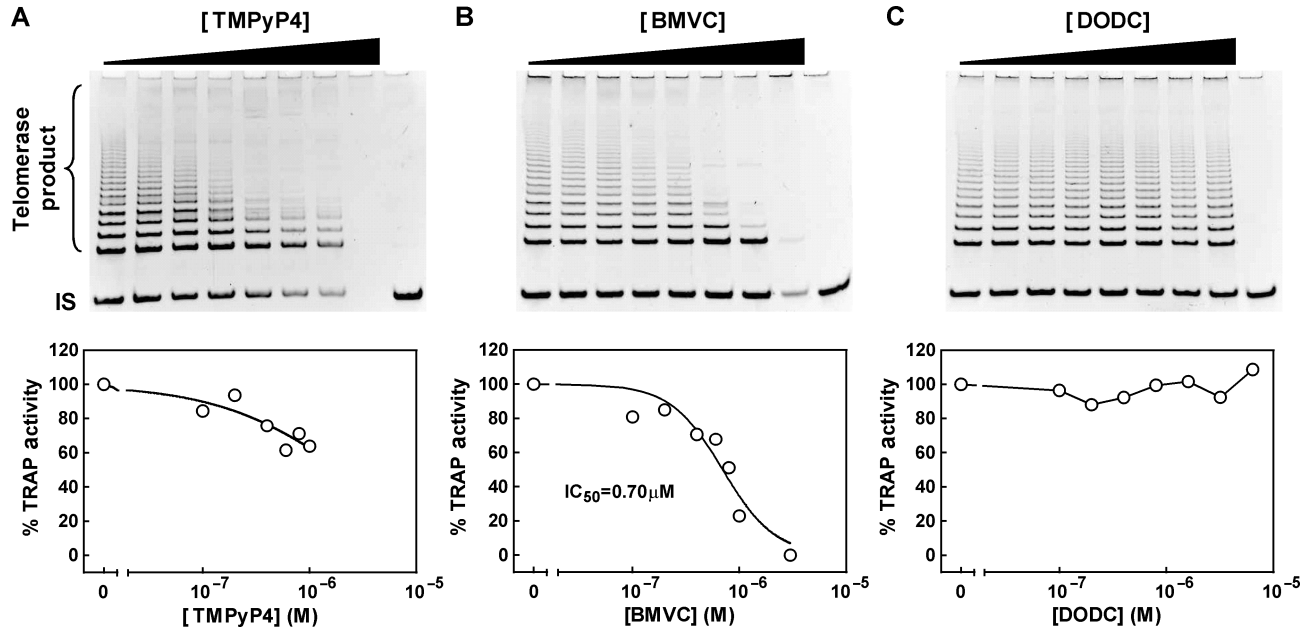

Figure 6. Effect of (**A**) TMPyP4, (**B**) BMVC and (**C**) DODC on telomerase activity. For each compound, the top panel shows ladder of amplified primer extension products stained with ethidium bromide; the last lane is the control using heat-inactivated telomerase in the absence of compound; the bottom panel shows the quantification of telomerase activity as percent of the control. IS indicates the bands of internal standard used to calibrate PCR efficiency.

used DNA polymerase stop assay (14), a quadruplex-forming sequence is placed in the middle of a linear template. Quadruplex stabilization stalls DNA synthesis at the quadruplex. On the one hand, inhibition in the linear part before the quadruplex via non-specific interaction may reduce DNA synthesis reaching the quadruplex and, as a result, leading to different exposures of quadruplex to polymerase in different treatments. On the other hand, such inhibition at the linear part on both sides of the quadruplex often produce non-specific arresting bands near the one produced by the quadruplex (10,14,39–45) that may be difficult to distinguish and sequencing gel may be needed for a better resolution (14,39–41,46). The purification of DNA substrate formed by annealing the ^{32}P-labeled primer to the template sequence by gel electrophoresis imposed extra work for

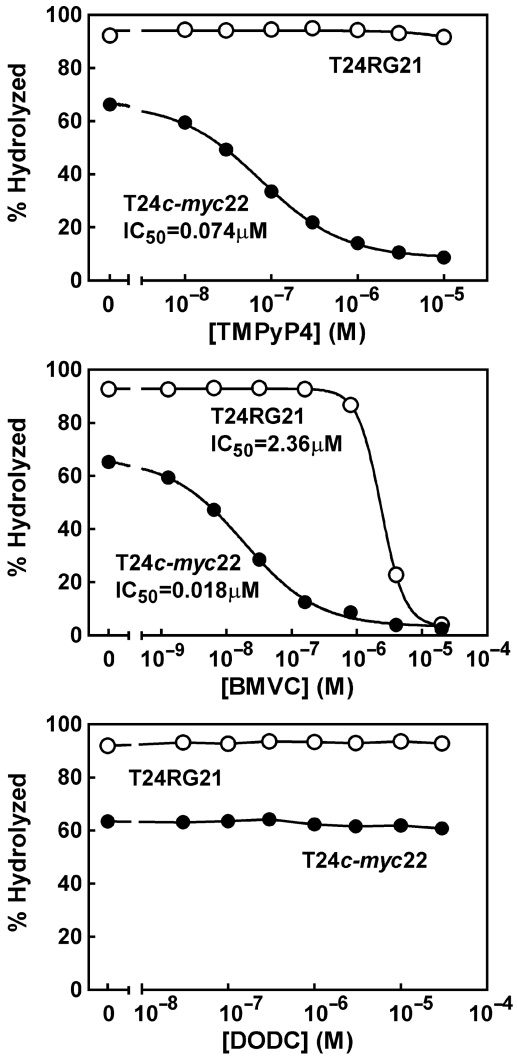

Figure 7. Effect of (**A**) TMPyP4, (**B**) BMVC and (**C**) DODC on the hydrolysis of the *c-myc* gene sequence T24c-myc22 (T_{24}GAGGGTGG GGAGGGTGGGGAAG, filled circles) and T24RG21 (open circles) by exonuclease I. Assays were carried out in buffer containing 2.5 mM KCl, 147.5 mM LiCl and the indicated compound at various concentrations. Results represent the mean of two hydrolysis experiments with range.

this assay (10,37,41,46). A TRAP-based method has been used for analyzing quadruplex stabilization specific to telomeric DNA (16). As is demonstrated in our Figure 6A, the inhibition on PCR may render the assay impractical for certain compounds like TMPyP4. Our data on the BMVC (Figure 4B) also indicates that the TRAP assay may not distinguish if the inhibition is on quadruplex or single-stranded substrate. In the PCR stop assay (15), quadruplex stabilization reduces primer annealing thus decreases PCR efficiency. Non-specific interactions may also have the same effect. Besides, it may also encounter similar problem as the TRAP assay since both of them involve PCR. Cationic molecules may interact with negative DNA molecules in a structure-independent manner as exemplified by the results shown in Figure 4B and D.

Our exonuclease assay provides an alternative method to evaluate quadruplex stabilization for, in principle, any quadruplex-forming sequence. Similar to the DNA polymerase stop assay, the TRAP method and the PCR stop assay, our method is applicable to intramolecular quadruplexes. It is simple, intuitive and easy to interpret. By running independent hydrolysis of two oligonucleotides, the assay avoids possible cross-interferences arising from interactions with the two forms of structures. Moreover, the assay easily clarifies contributions of inhibition from different sources (Figure 1), so one can quantify the effect of salt, quadruplex-relevant and quadruplex-irrelevant effects of small molecules separately. Such information is important in predicting the *in vivo* consequence of small molecules. For example, our data on TMPyP4 (Figure 3) and BMVC (Figure 4) revealed that the former was more specific and had little effect on the single-stranded DNA while the BMVC had a dramatic effect. These data suggest that, when applied *in vivo*, TMPyP4 may be less toxic than BMVC. In intracellular environment, quadruplex is already stabilized by ~150 mM of K^+. For intracellular applications, a compound will have effect only when it can further stabilize quadruplex in the presence of physiological concentration of K^+. The different hydrolysis of the two substrates in the absence of compounds (Figure 1B) reflects the quadruplex stabilization by K^+ in the buffer so one can easily judge, by comparing I_{SC} with I_{SS}, how a compound stabilizes quadruplex in addition to that already achieved by the K^+. This information may be particularly useful for predicting the effect under *in vivo* conditions where K^+ is present. The ratio of I_{SC}/I_{SS} can be used for calibration between assays and comparison between different compounds. The optimal concentration can also be determined at which a compound can reach maximal stabilization on quadruplex with minimal effect on single-stranded DNA.

From Figure 1B, it can be seen that the majority of quadruplex substrate has to be hydrolyzed in the absence of small molecules to allow the inhibition by small molecules to be detected. The hydrolysis in the assay is affected by the concentration of small molecules, K^+, exonuclease I and reaction time. While the concentration of small molecules is a variable to be analyzed, the other three parameters can be adjusted to optimize the assay condition. In practice, assay conditions can be determined as follows. First of all, 150 mM of K^+ should be preferentially used because it is the physiological concentration of K^+ inside animal cells. Then the concentration of exonuclease I or reaction time can be adjusted to digest appropriate amount of the quadruplex-forming substrate (~70% in our assays) in the absence of small molecules. Quadruplexes formed by certain sequences like *c-myc* can be very stable and resistant to exonuclease hydrolysis in 150 mM K^+ even in the absence of small molecules. In this case, lower K^+ concentration may be used to reduce quadruplex stability to elevate the hydrolysis of quadruplex-forming substrate to the desired degree. With fixed concentrations of exonuclease I and reaction time, one can carry out hydrolysis at various concentrations of K^+. The proper

K^+ concentration can then be found from the K^+ concentration-dependent hydrolysis curve. In the present work, the reaction products were analyzed by gel electrophoresis. Because there are only two bands, the analysis can be easily carried out on HPLC or capillary electrophoresis for high-throughput and large-scale screening.

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Conflict of interest statement. None declared.

REFERENCES

1. Shay,J.W. and Wright,W.E. (2005) Senescence and immortalization: role of telomeres and telomerase. *Carcinogenesis*, **26**, 867–874.
2. Hardin,C.C., Perry,A.G. and White,K. (2001) Thermodynamic and kinetic characterization of the dissociation and assembly of quadruplex nucleic acids. *Biopolymers*, **56**, 147–194.
3. Fletcher,T.M., Sun,D., Salazar,M. and Hurley,L.H. (1998) Effect of DNA secondary structure on human telomerase activity. *Biochemistry*, **37**, 5536–5541.
4. Zahler,A.M., Williamson,J.R., Cech,T.R. and Prescott,D.M. (1991) Inhibition of telomerase by G-quartet DNA structures. *Nature*, **350**, 718–720.
5. Sun,D., Thompson,B., Cathers,B.E., Salazar,M., Kerwin,S.M., Trent,J.O., Jenkins,T.C., Neidle,S. and Hurley,L.H. (1997) Inhibition of human telomerase by a G-quadruplex-interactive compound. *J. Med. Chem.*, **40**, 2113–2116.
6. Jing,N., Sha,W., Li,Y., Xiong,W. and Tweardy,D.J. (2005) Rational drug design of G-quartet DNA as anti-cancer agents. *Curr. Pharm. Des.*, **11**, 2841–2854.
7. Neidle,S. and Read,M.A. (2001) G-quadruplexes as therapeutic targets. *Biopolymers*, **56**, 195–208.
8. Rosu,F., De Pauw,E., Guittat,L., Alberti,P., Lacroix,L., Mailliet,P., Riou,J.F. and Mergny,J.L. (2003) Selective interaction of ethidium derivatives with quadruplexes: an equilibrium dialysis and electrospray ionization mass spectrometry analysis. *Biochemistry*, **42**, 10361–10371.
9. Teulade-Fichou,M.P., Carrasco,C., Guittat,L., Bailly,C., Alberti,P., Mergny,J.L., David,A., Lehn,J.M. and Wilson,W.D. (2003) Selective recognition of G-quadruplex telomeric DNA by a bis(quinacridine) macrocycle. *J. Am. Chem. Soc.*, **125**, 4732–4740.
10. Seenisamy,J., Bashyam,S., Gokhale,V., Vankayalapati,H., Sun,D., Siddiqui-Jain,A., Streiner,N., Shin-Ya,K., White,E. *et al.* (2005) Design and synthesis of an expanded porphyrin that has selectivity for the c-MYC G-quadruplex structure. *J. Am. Chem. Soc.*, **127**, 2944–2959.
11. Wang,P., Ren,L., He,H., Liang,F., Zhou,X. and Tan,Z. (2006) A phenol quaternary ammonium porphyrin as a potent telomerase inhibitor by selective interaction with quadruplex DNA. *Chembiochem*, **7**, 1155–1159.
12. Gurzadyan,G.G. (2002) Excited singlet state and photoionization of 8-methoxypsoralen. Picosecond transient absorption study. *Photochem. Photobiol. Sci.*, **1**, 757–762.
13. Mergny,J.L., Lacroix,L., Teulade-Fichou,M.P., Hounsou,C., Guittat,L., Hoarau,M., Arimondo,P.B., Vigneron,J.P., Lehn,J.M. *et al.* (2001) Telomerase inhibitors based on quadruplex ligands selected by a fluorescence assay. *Proc. Natl Acad. Sci. USA*, **98**, 3062–3067.
14. Han,H., Hurley,L.H. and Salazar,M. (1999) A DNA polymerase stop assay for G-quadruplex-interactive compounds. *Nucleic Acids Res.*, **27**, 537–542.
15. Lemarteleur,T., Gomez,D., Paterski,R., Mandine,E., Mailliet,P. and Riou,J.F. (2004) Stabilization of the c-myc gene promoter quadruplex by specific ligands' inhibitors of telomerase. *Biochem. Biophys. Res. Commun.*, **323**, 802–808.
16. Gomez,D., Mergny,J.L. and Riou,J.F. (2002) Detection of telomerase inhibitors based on G-quadruplex ligands by a modified telomeric repeat amplification protocol assay. *Cancer Res.*, **62**, 3365–3368.
17. Kan,Z.Y., Yao,Y., Wang,P., Li,X.H., Hao,Y.H. and Tan,Z. (2006) Molecular crowding induces telomere G-quadruplex formation under salt-deficient conditions and enhances its competition with duplex formation. *Angew. Chem. Int. Ed. Engl.*, **45**, 1629–1632.
18. Brody,R.S., Doherty,K.G. and Zimmerman,P.D. (1986) Processivity and kinetics of the reaction of exonuclease I from *Escherichia coli* with polydeoxyribonucleotides. *J. Biol. Chem.*, **261**, 7136–7143.
19. Zhao,Y., Kan,Z.Y., Zeng,Z.X., Hao,Y.H., Chen,H. and Tan,Z. (2004) Determining the folding and unfolding rate constants of nucleic acids by biosensor. Application to telomere G-quadruplex. *J. Am. Chem. Soc.*, **126**, 13255–13264.
20. Kim,N.W. and Wu,F. (1997) Advances in quantification and characterization of telomerase activity by the telomeric repeat amplification protocol (TRAP). *Nucleic Acids Res.*, **25**, 2595–2597.
21. Ambrus,A., Chen,D., Dai,J., Bialis,T., Jones,R.A. and Yang,D. (2006) Human telomeric sequence forms a hybrid-type intramolecular G-quadruplex structure with mixed parallel/antiparallel strands in potassium solution. *Nucleic Acids Res.*, **34**, 2723–2735.
22. Xu,Y., Noguchi,Y. and Sugiyama,H. (2006) The new models of the human telomere d[AGGG(TTAGGG)(3)] in K(+) solution. *Bioorg. Med. Chem.*, **14**, 5584–5591.
23. Luu,K.N., Phan,A.T., Kuryavyi,V., Lacroix,L. and Patel,D.J. (2006) Structure of the human telomere in K(+) solution: an intramolecular (3 + 1) G-quadruplex scaffold. *J. Am. Chem. Soc.*, **128**, 9963–9970.
24. Han,F.X., Wheelhouse,R.T. and Hurley,L.H. (1999) Interactions of TMPyP4 and TMPyP2 with quadruplex DNA. Structural basis for the differential effects on telomerase inhibition. *J. Am. Chem. Soc.*, **121**, 3561–3570.
25. Yamashita,T., Uno,T. and Ishikawa,Y. (2005) Stabilization of guanine quadruplex DNA by the binding of porphyrins with cationic side arms. *Bioorg. Med. Chem.*, **13**, 2423–2430.
26. Mita,H., Ohyama,T., Tanaka,Y. and Yamamoto,Y. (2006) Formation of a complex of 5,10,15,20-tetrakis(N-methylpyridinium-4-yl)-21H,23H-porphyrin with G-quadruplex DNA. *Biochemistry*, **45**, 6765–6772.
27. Gabelica,V., Baker,E.S., Teulade-Fichou,M.P., De Pauw,E. and Bowers,M.T. (2007) Stabilization and structure of telomeric and c-myc region intramolecular G-quadruplexes: the role of central cations and small planar ligands. *J. Am. Chem. Soc.*, **129**, 895–904.
28. Shi,D.F., Wheelhouse,R.T., Sun,D. and Hurley,L.H. (2001) Quadruplex-interactive agents as telomerase inhibitors: synthesis of porphyrins and structure-activity relationship for the inhibition of telomerase. *J. Med. Chem.*, **44**, 4509–4523.
29. Chang,C.C., Kuo,I.C., Lin,J.J., Lu,Y.C., Chen,C.T., Back,H.T., Lou,P.J. and Chang,T.C. (2004) A novel carbazole derivative, BMVC: a potential antitumor agent and fluorescence marker of cancer cells. *Chem. Biodivers.*, **1**, 1377–1384.
30. Chen,Q., Kuntz,I.D. and Shafer,R.H. (1996) Spectroscopic recognition of guanine dimeric hairpin quadruplexes by a carbocyanine dye. *Proc. Natl Acad. Sci. USA*, **93**, 2635–2639.
31. Fu,W., Begley,J.G., Killen,M.W. and Mattson,M.P. (1999) Anti-apoptotic role of telomerase in pheochromocytoma cells. *J. Biol. Chem.*, **274**, 7264–7271.
32. Li,C.P., Huang,J.H., Chang,A.C., Hung,Y.M., Lin,C.H., Chao,Y., Lee,S.D., Whang-Peng,J. and Huang,T.S. (2004) A G-quadruplex ligand 3,3′-diethyloxadicarbocyanine iodide induces mitochondrion-mediated apoptosis but not decrease of telomerase activity in nasopharyngeal carcinoma NPC-TW01 cells. *Pharm. Res.*, **21**, 93–100.
33. Kerwin,S.M. (2000) G-quadruplex DNA as a target for drug design. *Curr. Pharm. Des.*, **6**, 441–478.

34. Huppert,J.L. and Balasubramanian,S. (2005) Prevalence of quadruplexes in the human genome. *Nucleic Acids Res.*, **33**, 2908–2916.
35. Todd,A.K., Johnston,M. and Neidle,S. (2005) Highly prevalent putative quadruplex sequence motifs in human DNA. *Nucleic Acids Res.*, **33**, 2901–2907.
36. Hurley,L.H. (2001) Secondary DNA structures as molecular targets for cancer therapeutics. *Biochem. Soc. Trans.*, **29**, 692–696.
37. Seenisamy,J., Rezler,E.M., Powell,T.J., Tye,D., Gokhale,V., Joshi,C.S., Siddiqui-Jain,A. and Hurley,L.H. (2004) The dynamic character of the G-quadruplex element in the c-MYC promoter and modification by TMPyP4. *J. Am. Chem. Soc.*, **126**, 8702–8709.
38. Mergny,J.L., Phan,A.T. and Lacroix,L. (1998) Following G-quartet formation by UV-spectroscopy. *FEBS Lett.*, **435**, 74–78.
39. Kerwin,S.M., Sun,D., Kern,J.T., Rangan,A. and Thomas,P.W. (2001) G-quadruplex DNA binding by a series of carbocyanine dyes. *Bioorg. Med. Chem. Lett.*, **11**, 2411–2414.
40. Duan,W., Rangan,A., Vankayalapati,H., Kim,M.Y., Zeng,Q., Sun,D., Han,H., Fedoroff,O.Y., Nishioka,D. *et al.* (2001) Design and synthesis of fluoroquinophenoxazines that interact with human telomeric G-quadruplexes and their biological effects. *Mol. Cancer Ther.*, **1**, 103–120.
41. De Armond,R., Wood,S., Sun,D., Hurley,L.H. and Ebbinghaus,S.W. (2005) Evidence for the presence of a guanine quadruplex forming region within a polypurine tract of the hypoxia inducible factor 1alpha promoter. *Biochemistry*, **44**, 16341–16350.
42. Siddiqui-Jain,A., Grand,C.L., Bearss,D.J. and Hurley,L.H. (2002) Direct evidence for a G-quadruplex in a promoter region and its targeting with a small molecule to repress c-MYC transcription. *Proc. Natl Acad. Sci. USA*, **99**, 11593–11598.
43. Katahira,M., Fukuda,H., Kawasumi,H., Sugimura,T., Nakagama,H. and Nagao,M. (1999) Intramolecular quadruplex formation of the G-rich strand of the mouse hypervariable minisatellite Pc-1. *Biochem. Biophys. Res. Commun.*, **264**, 327–333.
44. Kim,M.Y., Duan,W., Gleason-Guzman,M. and Hurley,L.H. (2003) Design, synthesis, and biological evaluation of a series of fluoroquinoanthroxazines with contrasting dual mechanisms of action against topoisomerase II and G-quadruplexes. *J. Med. Chem.*, **46**, 571–583.
45. Rezler,E.M., Seenisamy,J., Bashyam,S., Kim,M.Y., White,E., Wilson,W.D. and Hurley,L.H. (2005) Telomestatin and diseleno sapphyrin bind selectively to two different forms of the human telomeric G-quadruplex structure. *J. Am. Chem. Soc.*, **127**, 9439–9447.
46. Sun,D., Guo,K., Rusche,J.J. and Hurley,L.H. (2005) Facilitation of a structural transition in the polypurine/polypyrimidine tract within the proximal promoter region of the human VEGF gene by the presence of potassium and G-quadruplex-interactive agents. *Nucleic Acids Res.*, **33**, 6070–6080.