Mechanism of Action of G-Quadruplex-Forming Oligonucleotide Homologous to the Telomere Overhang in Melanoma

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T-oligo, a guanine-rich oligonucleotide homologous to the 3′-telomeric overhang of telomeres, elicits potent DNA-damage responses in melanoma cells; however, its mechanism of action is largely unknown. Guanine-rich oligonucleotides can form G-quadruplexes (G4), which are stabilized by the hydrogen bonding of guanine residues. In this study, we confirmed the G4-forming capabilities of T-oligo using nondenaturing PAGE, nuclear magnetic resonance, and immunofluorescence. Using an anti-G-quadruplex antibody, we showed that T-oligo can form G4 in the nuclei of melanoma cells. Furthermore, using DNase I in a nuclease degradation assay, G4-T-oligo was found to be more stable than single-stranded T-oligo. G4-T-oligo had decreased anti-proliferative effects compared with single-stranded T-oligo. However, G4-T-oligo has similar cellular uptake as single-stranded T-oligo, as shown by FACS analysis. Inhibition of JNK, which causes DNA damage-induced apoptosis, partially reversed the antiproliferative activity of T-oligo. T-oligo also inhibited mRNA expression of human telomerase reverse transcriptase, a catalytic subunit of telomerase that was reversed by JNK inhibition. Furthermore, two shelterin complex proteins TRF2/POT1 were found to be up-regulated and bound by T-oligo, suggesting that T-oligo may mediate dissociation of these proteins from the telomere overhang. These studies show that T-oligo can form a G-quadruplex and that the antitumor effects of T-oligo may be mediated through POT1/TRF2 and via human telomerase reverse transcriptase inhibition through JNK activation.


INTRODUCTION

Recently, telomeres have become an attractive target for anticancer therapeutics because of near-universal over-expression of telomerase in tumors, which endows malignant cells with immortality (Crees et al., 2014; Ruden and Puri, 2013). Guanine-rich oligonucleotides (GROs), including single-stranded (SS) telomere homolog oligonucleotides, are known to have potent anticancer effects when administered to several malignant cell types both in vitro and in vivo (Crees et al., 2014). Previous studies have shown that increasing the stability of telomeric G-quadruplexes (G4s), four-stranded DNA secondary structures stabilized by the sequential stacking of guanine quartets in a planar arrangement, using exogenous G4-stabilizing ligands induces dissociation of telomere-associated proteins TRF2 and POT1, which are critical for telomere stability (Gomez et al., 2006; Tahara et al., 2006). Growing evidence shows that G4 formation can stall the replication fork during DNA synthesis and inhibit telomere replication by preventing telomerase from binding to the telomere (Crees et al., 2014; Maizels, 2006). Therefore, further studies are required to understand the anticancer effects of GROs and the role of telomere-associated proteins in their mechanism of action.

One particularly well-studied 11-base T-oligo (dGTTAGGTTAG), which is homologous to the 3′-telomere overhang sequence, induces potent DNA damage responses (DDRs) in several cancers (Eller et al., 2002; Puri et al., 2004, 2014; Ruden and Puri, 2013; Wojdyla et al., 2014; Yaar et al., 2007) and has minimal or no toxicity on normal cells (Puri et al., 2004, 2014). T-oligo is also known to induce enhanced melanogenesis and also decreases chronic photodamage to human skin by increasing DNA repair after UVR (Arad et al., 2006). Moreover, T-oligo is a powerful modulator of pigmentation and growth of organ-cultured human scalp hair follicles, further emphasizing its role in the field of dermatology (Hadshiew et al., 2008). Our earlier studies indicated that SS-T-oligo may possibly recruit the shelterin complex, a group of six proteins regulating telomeric stability and homeostasis, away from the telomere, uncapping the telomere overhang and causing DDRs (Pitman et al., 2013). GROs such as T-oligo can also form G4s (Crees et al., 2014; Rankin et al., 2013; Ruden and Puri, 2013), and some
evidence suggests that G4 formation by GROs may mediate DDRs, presumably by altering telomere architecture (Crees et al., 2014; Rankin et al., 2013). However, the functional roles of these secondary structures in inducing DDRs have not been thoroughly described. Currently, there is no clear correlation between G4 formation and anticancer activity of GROs in terms of secondary structures (Page et al., 1999). Therefore, it is important to study G4-forming capabilities of GROs such as T-oligo to improve their design for cancer therapy. Further studies are required to understand the anticancer effects of T-oligo, including their relation to telomeres and potential use in melanoma treatment.

The purpose of this study was to determine the G4-forming potential of an oligonucleotide homologous to the telomere overhang, T-oligo, using nuclear magnetic resonance (NMR), the criterion standard in G4 detection (Adrian et al., 2012). G4 formation by T-oligo was also investigated inside the nucleus of melanoma cells using an antibody that detects G4s. We also show that the anticancer signaling mechanism of T-oligo involves human telomerase reverse transcriptase (hTERT) and JNK, which further suggests that T-oligo induces DDR signals through inhibition of telomerase and telomere-associated proteins.

RESULTS
Detection of G4-T-oligo by nondenaturing PAGE and NMR
The G4 structures were verified by loading 0.1 μg of the SS-T-oligo and G4-T-oligo into a 20% polyacrylamide gel under nondenaturing conditions. G4 formation by T-oligo was enhanced in the presence of 500 mmol/L KCl (Figure 1a), whereas the same concentration of NaCl produced negligible G4 content (Figure 1b). The extent of G4 formation depends significantly on the concentration of T-oligo present during thermal denaturation, which is expected for G4 formation because four strands must come together to form one complex (Figure 1c). Hence, increasing concentration of SS-T-oligo favors formation of more G4. NMR studies for T-oligo in KCl showed the presence of downfield imino proton resonances (9–14 ppm), which confirmed that T-oligo forms G4 structures (Figure 1d). These resonances were absent in NaCl (Figure 1e), which was consistent with nondenaturing PAGE experiments showing negligible G4 in NaCl.

Confirmation of G4 formation by T-oligo inside nuclei of melanoma cells
To confirm G4 formation by T-oligo, we used a specific G4-detecting antibody (BG4) (Biffi et al., 2013). Immunofluorescence studies using FITC-T-oligo and BG4 antibody confirmed the formation of G4 foci in the nuclei of melanoma cells after 4 hours of FITC-T-oligo treatment (Figure 2a). When melanoma cells were treated only with BG4 (Figure 2b) or FITC-C-oligo (Figure 2c), they exhibited minimal or no foci formation.

Antiproliferative activity, resistance to nuclease activity, and cellular uptake of SS-T-oligo and G4-T-oligo
The stability of G4 was examined by incubating G4 with DNase I for different time periods. G4-T-oligo partially resisted nuclease degradation by DNase I for up to 90
minutes (Figure 3a). Densitometry analysis after nuclease digestion showed a 52.6% decrease in T-oligo and a 20% decrease in G4-T-oligo, with a 96% increase in SS free T-oligo in the G4-T-oligo sample after 90 minutes of DNase I treatment (Figure 3a). These results indicate that G4-T-oligo is more resistant to nuclease treatment compared with SS-T-oligo and can be converted into SS-T-oligo. Furthermore, the results of antiproliferative activity of SS-T-oligo and

Figure 2. Detection of G4-structures formed by T-oligo in the nuclei of melanoma cells. (a) MM-AN cells were plated in eight-well glass chamber slides and treated with 40 μmol/L FITC-T-oligo for 4 hours. Cells were fixed, permeabilized, blocked, and incubated with BG4 for 2 hours at room temperature. Foci were observed in the nuclei using Cy3-conjugated anti-mouse secondary antibody (upper panel). A single cell observed under high magnification (bottom panel). A confocal microscope and its image analysis software were used. Top panel scale bar = 20 μm, bottom panel scale bar = 10 μm. (b) Melanoma cells treated with only BG4 antibody showed minimal number of foci. Scale bar = 20 μm. (c) Melanoma cells treated with FITC-C-oligo (negative control) showed no G4 foci formation. Scale bar = 5 μm. G4, G-quadruplex.
G4-T-oligo showed that G4-T-oligo was less effective (27.1–32.4%) than SS-T-oligo (42.8–61.6%) in inhibiting melanoma cell proliferation, as observed after MTT assay (Figure 3b). However, both SS-T-oligo and G4-T-oligo had comparable cellular uptake as measured by FACS analysis (Figure 3c). This suggests that the reduced antiproliferative activity of G4-T-oligo is not likely attributed to poor cellular uptake. This phenomenon could be attributable to reduction of SS-T-oligo in the G4 form and its ability to bind TRF2 and POT1, as described in the next section.

Interaction of T-oligo with telomere-associated proteins

TRF2 and POT1

Densitometry analysis after Western blotting showed that T-oligo treatment up-regulated TRF2 by 2.2- and 3.0-fold ($P < 0.01$) at 48 hours and 72 hours, respectively, and POT1 by 3.0-fold ($P < 0.02$) both at 48 and 72 hours in melanoma cells (Figure 4a–d), which may be a response to uncapped telomeres induced by T-oligo (Karlseder et al., 1999) and corroborates well with the hypothesis that T-oligo induces telomere dysfunction. Immunofluorescence studies confirmed up-regulation of TRF2 (2.4-fold) and POT1 (2.0-fold) after treatment with T-oligo (Figure 4g). After administration, T-oligo rapidly enters into the nucleus in melanoma cells. However, whether or not T-oligo binds to or associates with the telomere or its components is currently unknown. The predominant bands of TRF2 and POT1 in Western blot analysis of melanoma cell lysates after pulldown assay using T-oligo and streptavidin magnetic beads showed that T-oligo indeed interacted with these telomeric proteins (Figure 4e and f).

Antiproliferative effects of T-oligo through JNK

Western blot analysis showed up-regulation of both phosphorylated (p) JNK and total JNK by 4.0- and 2.0-fold, respectively, at 24 hours in T-oligo-treated melanoma cells (Figure 5a). JNK, an inducer of apoptosis, has previously been shown to be activated by G4-stabilizing ligands causing telomere dysfunction (Rankin et al., 2013). To further confirm the involvement of p-JNK in T-oligo-mediated apoptosis, we used a specific JNK inhibitor SP600125. Western blot analysis showed that T-oligo-mediated up-regulation of p-JNK was reversed in the presence of SP600125 (Figure 5b). The MTT assay results showed a 73.8% decrease in cell viability after 96 hours of T-oligo treatment alone in melanoma cells; however, cell viability was decreased to 71.8%, 55.2%, 45.8%, and 25.3% when SP600125 was present in concentrations of 4 μmol/L, 8 μmol/L, 10 μmol/L, and 12 μmol/L, respectively, compared with diluent (Figure 5c). Treatment with SP600125 alone had minimal effect on cell proliferation. These results show that the inhibition of JNK by SP600125 reversed the antiproliferative activity of T-oligo in a dose-dependent manner.

DOWN-REGULATION OF hTERT EXPRESSION BY T-OLIGO

To investigate the role of hTERT in T-oligo-induced melanoma cell death, the mRNA expression of hTERT was assessed using quantitative real-time reverse transcriptase (qRT)–PCR. The mRNA expression of hTERT was decreased by 50% after treatment with T-oligo in melanoma cells (Figure 5d). Because T-oligo increased JNK phosphorylation, it was speculated that the JNK pathway may be involved in T-oligo-mediated hTERT down-regulation. To test this hypothesis, we investigated the effect of the JNK inhibitor SP600125 on hTERT expression in T-oligo–treated MM-AN cells. Treatment with 12 μmol/L SP600125 in presence of T-oligo partially reversed the down-regulation of hTERT, because we observed a decrease of only 16% in hTERT expression compared with a 50% reduction by T-oligo treatment alone. SP600125 alone had no effect on hTERT expression (Figure 5d). The results suggest that activation of JNK may be required for hTERT down-regulation by T-oligo in melanoma cells.
DISCUSSION

The incidence of malignant melanoma has increased significantly during the past decade (Puri et al., 2007; Siegel et al., 2016). Metastatic melanoma is very challenging to treat because it is highly resistant to chemotherapy and is almost always fatal (Puri et al., 2004). Telomeres and telomerase have become popular targets for cancer therapeutics because of their involvement in the development of immortality in nearly 85–90% of all cancers (Cookson and Laughton, 2009; Ruden and Puri, 2013). A study by Eller et al. (1994) showed that thymidine dinucleotides and other oligonucleotides could mimic cellular responses to UV irradiation, including enhanced melanogenesis (Eller et al., 1994). Further studies using an oligonucleotide homologous to the telomere overhang called T-oligo (5'-GTTAGGGTTAG-3') have been shown to induce potent DDRs in several cancers, including melanoma (Eller et al., 2002; Puri et al., 2004; Rankin et al., 2008; Yaar et al., 2007).

In this study, we show that T-oligo forms stable, 4-stranded, intermolecular G4 structures in vitro in the presence of KCl using one-dimensional NMR spectra and nondenaturing PAGE. The downfield imino proton resonances (9–14 ppm) obtained in the NMR data for G4s correspond to each guanine residue that is exchanging protons with the solution at a particular frequency. The G4 formation by T-oligo was observed to be highly dependent on strand concentration as confirmed using nondenaturing PAGE and suggests the fourth order molecularity of a tetramolecular G4 (Mergny et al., 2005). Furthermore, to examine the G4 formation by T-oligo that may occur inside the cellular environment after internalization, a specific anti-G4 antibody, BG4, (Biffi et al., 2013) was used. Immunofluorescent foci specific to G4 structures after treatment with FITC-T-oligo were observed inside the nuclei of melanoma cells. These results suggest that T-oligo may spontaneously adopt a G4 structure at physiologic conditions inside the cells.

Furthermore, when MM-AN cells were treated with G4-T-oligo, its antiproliferative activity was significantly reduced compared with its SS counterpart, which showed higher antiproliferative effects in a dose-dependent manner. In contrast, there was no dose-dependent increase in antiproliferative activity of G4-T-oligo. However, FACS analysis showed that cellular uptake between SS-T-oligo and G4-T-oligo was comparable, suggesting that the reduced antiproliferative activity of G4-T-oligo was not attributed to differences in cellular uptake. One possible explanation for the reduced antiproliferative activity of G4-T-oligo may be that intermolecular G4 formation effectively reduces strand concentration compared with SS species, thereby reducing the number of strands that can elicit anticancer responses. The SS-T-oligo may also bind more effectively to TRF2 and POT1, inducing DNA damage and cell death, which is reduced in the case of G4-T-oligo. Moreover, G4-T-oligo was found to be more stable after DNase I digestion than SS-T-oligo and can be converted into SS-T-oligo by DNase I.

**Figure 4. Effects of T-oligo on TRF2 and POT1 in melanoma cells.** (a, b) MM-AN cells were treated with diluent, 40 μmol/L T-oligo, or C-oligo for 48–72 hours followed by Western blot. (c, d) Bar graphs showing densitometry analysis of TRF2 and POT1 bands. Fold change was relative to the diluent. TRF2, *P < 0.01; POT1, **P < 0.02. (e, f) Western blot for TRF2 and POT1 in melanoma cells after a pulldown assay using biotinylated T-oligo and C-oligo. (g) 20,000 MM-AN cells were plated per chamber of an eight-chamber slide and treated with 40 μmol/L T-oligo for 12 hours, followed by incubation with anti-TRF2 or anti-POT1 antibody. Up-regulation of TRF2 and POT1 was analyzed compared with diluent. Scale bar = 10 μm. hrs, hours.
bands of TRF2 and POT1 proteins in biotinylated T-oligo Rad50-MRE11-NBS1 complex (Zhu et al., 2000), ATM may play active roles in the cellular response to damaged or involved in DNA double-strand break recognition and repair co-localized with telomeres (Eller et al., 2006). Proteins (Nijjar et al., 2005). Our pulldown assay showed predominant resolution of the intermediates can lead to TRF2 accumulation (Opresko et al., 2002). Molecular defects that inhibit the reaction of the intermediates can lead to TRF2 accumulation (Nijjar et al., 2005). Our pulldown assay showed predominant bands of TRF2 and POT1 proteins in biotinylated T-oligo—treated melanoma cell lysates. It may be possible that TRF2 and POT1, which are known to bind to telomeric structures, also bind to T-oligo within the cell, inhibiting free TRF2 and POT1 from binding and leading to its accumulation. Additionally, T-oligo induced activation of SAPK/JNK in melanoma cells, corroborating previous studies performed in ovarian and lung cancer, which showed JNK activation by an intramolecular G4-forming GRO (Rankin et al., 2013; Sarkar and Faller, 2013). This was further confirmed using a specific JNK inhibitor SP600125, which diminished T-oligo—mediated JNK phosphorylation. Furthermore in cell proliferation assays, treatment of SP600125 with T-oligo in melanoma cells partially reversed the antiproliferative activity of T-oligo. Most malignant cells have high telomerase activity, but in most somatic cells it is barely detectable. Expression of hTERT, a catalytic subunit of telomerase, has been correlated with telomerase activity in cancer cells. T-oligo also inhibited hTERT expression in melanoma cells. It has been suggested that JNK activation may lead to down-regulation of hTERT (Tian et al., 2015). Thus, the antiproliferative activity of T-oligo could also be mediated through down-regulation of hTERT via the activation of JNK pathway.

In conclusion, we show that T-oligo forms tetrameric intermolecular G4s in vitro and also inside the cells, which can be detected by BG4. This study also shows that two GROs of the same sequence but different secondary structures (SS and G4), have significantly different cytotoxic activity, indicating an important structure-function relationship that could influence the design of future anticancer GROs. Furthermore, by showing the up-regulation of shelterin proteins TRF2 and POT1 after T-oligo treatment and pulldown assay using biotinylated T-oligo, we provide evidence that T-oligo interacts with the telomere and its associated proteins, causing telomere dysfunction in melanoma cells. It may also be possible that specific shelterin proteins dissociate from the telomere and bind to T-oligo, and thus melanoma cells produce more TRF2 and POT1 in an attempt to reverse telomere exposure. This study is an important step forward in understanding the anticancer effects of GROs and their relationship to secondary structures. Moreover, we also show that proteins inducing DDRs such as SAPK/JNK are activated after T-oligo treatment, leading to inhibition of hTERT expression in melanoma cells, providing insights into the mechanisms underlying the anticancer activity of T-oligo. Hence, this study opens avenues for future investigations and improvements in potential GRO therapies.
MATERIALS AND METHODS

Oligonucleotides

Oligonucleotides homologous (5'-dGTTAGGGTAG-3' [i.e., T11], and 5'-dCTAACGTCCTAC-3' [i.e., T12]) and complementary (5'-dCTAACCCCTAAC-3' [i.e., C-oligo]) to the 3'-telomere overhang sequence were obtained from Midland Certified Reagent Company (Midland, TX). T12 and C-oligo were used as positive and negative controls, respectively.

Antibodies

Antibodies for TRF2, p-SAPK/JNK, and total-SAPK/JNK were purchased from Cell Signaling (Danveres, MA). Anti-POT1 antibody was obtained from Abcam (Cambridge, MA). Anti-β-actin antibody was purchased from Sigma-Aldrich (St. Louis, MO). Anti-G4 antibody was purchased from Absolute Antibody (Oxford, UK).

Cell lines and cell culture

The MM-AN cell line was obtained by explant culture (Puri et al., 2004) and maintained at 37°C in MEM (Thermo Fisher Scientific, Pittsburg, PA) supplemented with 10% fetal bovine serum (ATCC Biologicals, Lawrenceville, GA) and 1% antibiotic/antimycotic (Invitrogen, Carlsbad, CA).

Cell line short tandem repeat profiling and authentication

DNA was extracted from MM-AN cells using QiAamp DNA mini kit (Qiagen, Valencia, CA), and short tandem repeat analysis was performed by a third party, Genetica DNA Laboratories (a LabCorp brand; Burlington, NC) using the PowerPlex16HS amplification kit (Promega Corporation, Fitchburg, WI). The short tandem repeat DNA profile of MM-AN cells was compared against known cell line reference databases (e.g., ATCC, DSMZ) and was found to be “unique” with no contamination of any other cell line or mycoplasma.

G4 formation and detection of G4s using NMR

To induce formation of G4, 200–2,000 μmol/L of oligonucleotides were diluted in Tris/EDTA buffer and incubated at 95°C for 5 minutes. The reaction was brought to 500 mmol/L NaCl or KCl and incubated at 60°C for 24 hours. The G4 structures were analyzed by loading 0.1 μg of samples on a 20% nondenaturing gel. NMR experiments were performed for T-oligo, C-oligo, and positive control T12 (0.5 mmol/L) at pH 6.5 using an 800 MHz AVANCE NMR spectrometer (Bruker, Billerica, MA) at 283–295 K. Full proton spectra of the annealed structures were recorded from 200–400 nm using a scan rate of 100 nm/minute in potassium or sodium solutions.

Nondenaturing PAGE

Samples were prepared in a Tris/borate/EDTA buffer containing 100 mmol/L NaCl or KCl at pH 7.0 and 1x agarose loading dye and then loaded (0.1 μg) into a 20% polyacrylamide gel. Electrophoresis was performed at room temperature for 2.5 hours at 100 V in Tris/borate/EDTA buffer containing 100 mmol/L KCl or NaCl. The gel was stained with 1x SYBR Gold for 30 minutes, and DNA was detected by intercalation-induced fluorescence (excitation = 495 nm, emission = 537 nm) with a ChemiDoc-It TS2 imaging system (UVP, Upland, CA).

MTT cell viability assay

A total of 5,000 MM-AN cells/well were plated in replicates of six in 96-well plates. Cells were treated with T-oligo at concentrations (10–40 μmol/L) found to be nontoxic and maximally effective for 4 hours, after which MTT assay was performed, as described previously (Uppada et al., 2014). For G4 studies, cells were treated with G4-T-oligo (10–40 μmol/L) prepared in the presence of 500 mmol/L KCl. For JNK inhibition, cells were treated with 10 μmol/L SP600125 (Sigma-Aldrich, St. Louis, MO) for 96 hours in the presence of absence of T-oligo. Percentage cell viability was determined relative to the control.

Nuclease digestion

The SS and G4-T-oligo were incubated with DNase I (5 U) for 0–90 minutes at 37°C. After incubation at 60°C for 10 minutes, the reaction was quenched with 2.5 mmol/L EDTA. Samples were analyzed on a 20% nondenaturing gel, which was stained in 1x SYBR Gold (Invitrogen, Carlsbad, CA) for 40 minutes and visualized on a transilluminator using the SYBR Gold filter settings.

Immunoblotting

MM-AN cells were grown in MEM and were treated with 40 μmol/L T-oligo, C-oligo, or diluent for 24–72 hours for lystate collection. For JNK inhibition, cells were treated with 10 μmol/L of SP600125 for 24 hours in the presence or absence of T-oligo. Immunoblotting was performed using antibodies against POT1, TRF2, total-JNK, and p-JNK, as described previously (Uppada et al., 2014). Immunoblots were developed using ECL chemiluminescence kit (Thermo Fisher Scientific, Rockford, IL). Densitometry analysis was performed using ImageJ software (National Institutes of Health, Bethesda, MD).

qRT-PCR

Total RNA was extracted using Qiagen Rneasy Mini Kit (Qiagen, Valencia, CA). qRT-PCR was performed using SuperScript III Platinum SYBR-Green One-Step Master-Mix on 7300 qRT-PCR system (Applied Biosystems, Carlsbad, CA). Specific hTERT primers (forward, 5'-GGCACACGTGCTTCTTCG-3'; reverse, 5'-GGTGAACCTCGTAAGTTGTTTGATGCAA-3') and GAPDH primers (forward, 5'-TTCGGAATGGACCCCTTCA-3'; reverse, 5'-CGCCCCACTTGATTTTGGA-3') were used. The expression of the hTERT gene was normalized with GAPDH levels. The fold changes were calculated using 2(-ΔΔCt) method.

Immunofluorescence

MM-AN cells (20,000 cells/well) were plated in eight-chamber slides and allowed to adhere overnight. For G4 detection, cells were treated with 40 μmol/L FITC-T-oligo for 4 hours and incubated with a specific anti-G4 antibody (BG4) for 2 hours at room temperature. For visualization of TRF2 and POT1 up-regulation, melanoma cells were treated with 40 μmol/L T-oligo for 12 hours and incubated with anti-TRF2 or anti-POT1 for 2 hours at room temperature. Cells were then incubated with Cy3 conjugated secondary antibody, and images were captured using the Olympus Fv10i Fluoview confocal microscope (Olympus, Tokyo, Japan).

Pulldown assay

MM-AN cells (1.2 × 10^6) were plated in 100-mm tissue culture dishes and allowed to grow for 24 hours. Cells were treated with diluent, biotinylated T-oligo (40 μmol/L), and biotinylated C-oligo (40 μmol/L) for 6 hours and were lysed. Each cell lystate (1,000 μg) was incubated with 50 μl of streptavidin magnetic beads (Thermo Fisher Scientific) for immunoprecipitation for 1 hour with constant rocking. The beads were washed twice with immunoprecipitation lysis buffer (Thermo Fisher Scientific) and once with distilled water. Proteins were eluted with 50 μl of low-pH Elution Buffer (Thermo Fisher Scientific), and 10 μl of the eluant was analyzed with Western blotting using anti-TRF2 and anti-POT1 antibodies.
Intracellular uptake of FITC-labeled T-oligo using FACS analysis

The uptake of FITC-labeled SS and G4-T-oligo by MM-AN cells was assessed using FACS. MM-AN cells (1.5 × 10^5) in MEM were plated in 35-mm cell culture dishes. After 24 hours, the cells were treated with 40 μmol/L FITC-T-Toligo (SS or G4) for 4 hours. Cells were then washed twice with Hank’s Balanced Salt Solution (Invitrogen, Carlsbad, CA), trypsinized, and pelleted. 1 × 10^4 cells were then analyzed on a BD FACS device (ImmunoCytometric Systems, San Jose, CA). The data were analyzed using Cell Quest analysis software.

Statistical analysis

All experiments were performed in triplicate. Statistical analyses were performed using Student *t* tests. *P* < 0.05 was considered statistically significant for all the experiments.

CONFLICT OF INTEREST

The authors state no conflict of interest.

REFERENCES


