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Conformation-Dependent Formation of the G[8−5]U Intrastrand Cross-Link in 5-Bromouracil-Containing G-Quadruplex DNA Induced by UVA Irradiation†

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ABSTRACT: G-quadruplex motifs are known to be present in telomeres of human and other organisms. Recent bioinformatic studies also revealed the widespread existence of these motifs in promoter regions of human genes. Treatment of cultured cells with 5-bromo-2′-deoxyuridine (^{Br}dU) is known to result in the substitution of DNA thymidine with ^{Br}dU; such replacement has been shown to sensitize cells to killing induced by UV light. Our previous studies revealed that the exposure of ^{Br}dU-carrying duplex DNA or ^{Br}dU-treated MCF-7 cells to UVB light could lead to the facile formation of intrastrand cross-link products initiated from ^{Br}dU. Here we found that the exposure of ^{Br}dU-bearing G-quadruplex DNA to UVA light could also give rise to the efficient formation of the G[8−5]U intrastrand cross-link, where the C8 of guanine in the external G-tetrad is covalently linked with the C5 of its adjacent 3′ uracil in the loop region. In addition, the yield for the cross-link product is dependent on the conformation of the G-quadruplex. Together, the formation of intrastrand cross-link in G-quadruplex motifs may account for the photocytotoxic effect induced by ^{Br}dU incorporation, and the ^{Br}dU-mediated photo-cross-linking may constitute a useful method for monitoring the different conformations of G-quadruplex folding.

The G-quadruplex structure is comprised of stacked G-tetrads, which are held together by a network of Hoogsteen hydrogen bonds and stabilized by monovalent cations like Na^+ and K^+ (*1*). Although *in vitro* formation of the G-quardruplex has been known for decades (*1*), substantial recent interest has been focused on the potential formation and function of this nucleic acid structure *in vivo*. For instance, the telomere, a region of repetitive DNA sequences at the end of chromosomes, has been shown to adopt G-quadruplex structures which can protect chromosomes against gene erosion and nuclease attack (*2*−*4*). In addition, the promoter regions of genes were found to be significantly enriched in G-quadruplex motifs relative to the remainder of the genome; more than 40% of human genes contain at least one G-quadruplex motif in their promoters (*5*), and G-quadruplex motifs in promoters may play a significant role in gene regulation (*5*−*7*).

G-quadruplex motifs exist in a variety of structural forms depending on their sequences and the identities of cations present. For example, the human telomere sequence, which consists of repeating d(TTAGGG), assumes a basket-type structure with one diagonal and two lateral loops in Na^+ solution (*8*), whereas in K^+ solution the favorable structure is a hybrid type with one double-chain reversal and two lateral loops (Figure 1a) (*9*−*12*).

It was observed several decades ago that the treatment of cells with 5-bromo-2′-deoxyuridine (^{Br}dU) could result in the incorporation of the modified nucleoside into genomic DNA, which replaces its isosteric thymidine (*13*, *14*). In addition, such treatment could render cells more sensitive toward killing induced by exposure to UV light or ionizing radiation (*13*, *15*). Our previous studies demonstrated that the exposure of 5-bromouracil-bearing duplex DNA and ^{Br}dU-treated MCF-7 human breast cancer cells to UVB light could lead to the facile formation of intrastrand cross-link products where the C5 of uracil is covalently attached with its neighboring guanine or adenine (*16*, *17*). In addition, the types and yields of the intrastrand cross-link products formed are affected by nucleobase stacking (*16*). However, it has not been assessed how G-quadruplex folding affects the UV light-induced formation of intrastrand cross-link product from ^{Br}dU.

In this study, we investigated the photoreactivity of 5-bromouracil-containing G-quadruplex DNA to explore the conformation-dependent intrastrand cross-link product formation in different loops of G-quadruplex upon irradiation with 365 nm UVA light. UVA light was employed for its relatively low energy compared to UVB light used in our previous studies (*16*, *17*) so that it can prevent the G-quadruplex structure from being destroyed during irradiation.

MATERIALS AND METHODS

Materials. All chemicals unless otherwise specified were obtained from Sigma-Aldrich (St. Louis, MO). Reagents used for solid-phase DNA synthesis were purchased from Glen Research Inc. (Sterling, VA). All unmodified oligodeoxyribonucleotides (ODNs) used in this study were from Integrated DNA Technologies (Coralville, IA).

ODNs containing a ^{Br}dU were synthesized at 1 μmol scale on a Beckman Oligo 1000S DNA synthesizer (Fullerton, CA) by using the commercially available phosphoramidite building block of ^{Br}dU (Glen Research Inc.). The nucleobase deprotection was carried out in 29% ammonia at room temperature for 72 h; the

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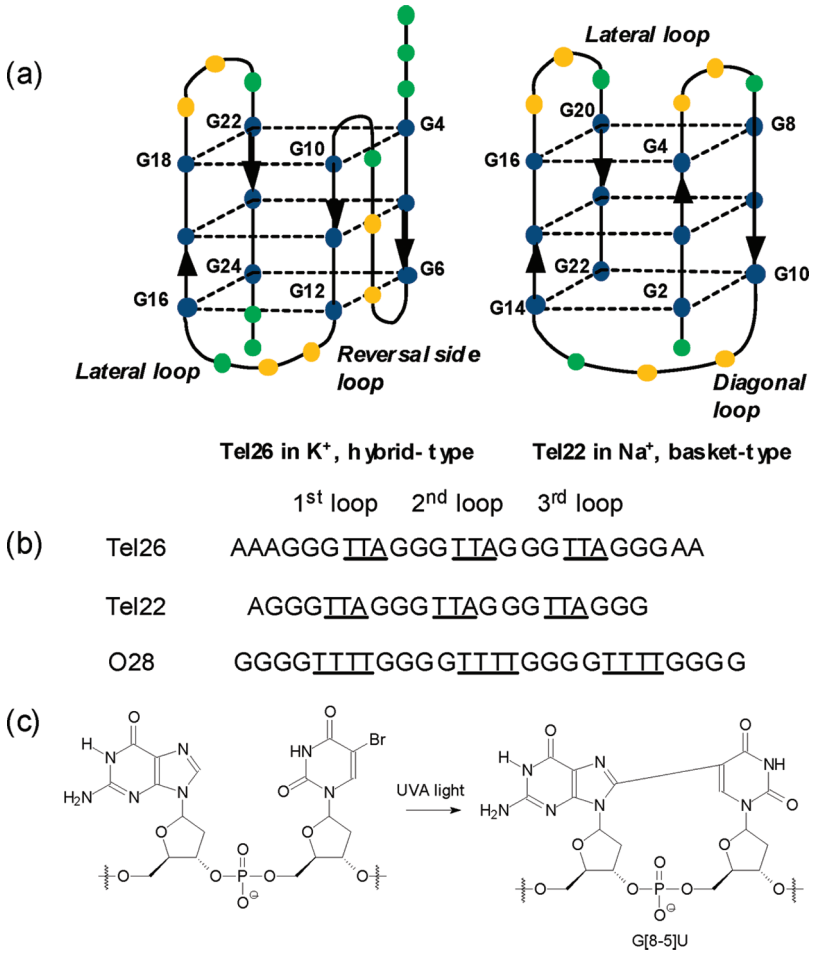

FIGURE 1: (a) Basket and hybrid forms of human telomeric DNA that could be adopted by Tel22 in Na^+ solution and Tel26 in K^+ solution, respectively. (b) The nucleobases located in the three loops of the G-quadruplex are underlined. (c) Photochemical cross-linking of 5-bromouracil with its neighboring guanine to give the G[8−5]U intrastrand cross-link.

deprotection at room temperature was necessary for minimizing the decomposition of the halogenated nucleoside. The identities of all ^{Br}dU-containing ODNs were confirmed by ESI-MS and MS/MS. Standard d(G[8−5]U) was synthesized previously (*16*).

Preparation of G-Quadruplexes. A 25 μM unmodified or ^{Br}dU-containing ODN was annealed in a 200 μL solution containing 25 mM potassium/sodium phosphate (pH 7.0) and 75 mM potassium/sodium chloride, and the formation of the G-quadruplex structure was confirmed by circular dichroism (CD) spectroscopy measurement.

UVA Irradiation. UVA irradiation was carried out with two 15 W Spectroline light tubes with emitting wavelength centered at 365 nm (Spectronics Corp., Westbury, NY). A 200 μL solution of the annealed ^{Br}dU-containing ODN (25 μM) was dispersed in the cap of a 1.5 mL centrifuge tube and irradiated on a bed of ice for 1 h at a distance of 4.5 cm from the UV lamp. The irradiation dose was 0.25 J/cm^2, which was measured using a Mannix UV-340 light meter (Mannix Instrument Inc., New York, NY).

CD Spectroscopy. CD spectra of ODNs were recorded on a Jasco J-815 spectrometer (Jasco, Easton, MD). A quartz cell from Starna (Atascadero, CA) with 1 mm optical path length was used for the measurement. The scanning speed was 100 nm/min with 1 s response time. The CD spectra reported in this paper were averaged from signal of three repetitive measurements between 200 and 320 nm at room temperature. Spectra were baseline-corrected, and the signal contributions of the buffer were subtracted.

Enzymatic Digestion. The enzymatic digestion was performed following our previously reported method (*16*, *18*). Briefly, a 5 nmol photoirradiation mixture was desalted by HPLC and dried by Speed-vac. One unit of nuclease P1, 0.005 unit of calf spleen phosphodiesterase, and 10 μL of 0.3 M zinc acetate (pH 5.0) were added, and the digestion was continued at 37 °C for 6 h. To the resulting solution were subsequently added 10 units of alkaline phosphatase, 0.01 unit of snake venom phosphodiesterase, and 20 μL of 0.5 M Tris-HCl solution (pH 8.9). The digestion was continued at 37 °C for another 6 h. In this respect, we showed previously that the same digestion procedures could allow for the release of ~88% of a guanine−thymine intrastrand cross-link from ODNs as the lesion-containing dinucleoside monophosphate (*18*); because of the structure similarity between the guanine−thymine and G[8−5]U cross-link examined in the present study, the efficiency for the enzymatic release of the latter cross-link should be similar. The digestion mixture was extracted twice with an equal volume of chloroform to remove the enzymes. The aqueous layer was dried, redissolved in water, and subjected to liquid chromatography−tandem mass spectrometry (LC-MS/MS) analysis.

HPLC. A 4.6 × 250 mm Apollo C18 column (5 μm in particle size, 300 Å) was used for the purification of synthetic ODNs and desalting of synthetic and UVA-irradiated ODNs. For ODN purification, a solution of 50 mM triethylammonium acetate (TEAA, solution A) and a mixture of 50 mM TEAA and methanol (40/60, v/v, solution B) were used as mobile phases.

A gradient of 0−30% B in 5 min, 30−70% B in 40 min, and 70−100% B in 5 min was employed; the flow rate was 0.8 mL/min. The desalting of ODNs was carried out by loading the ODN samples to the column, washing the column with water for 20 min, and eluting the ODNs from the column with a mixture of water and methanol (50/50 v/v); the flow rate was 0.6 mL/min.

LC-MS/MS Analysis. Quantification of cross-link products in the UVA-irradiated ODN digestion mixture was performed by online capillary HPLC-ESI-MS/MS analysis using an Agilent 1200 capillary HPLC pump (Agilent Technologies, Santa Clara, CA) interfaced with an LTQ linear ion trap mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The system was set up for monitoring the fragmentation of the $[M + H]^+$ ions of d(G[8−5]U) (*m*/*z* 556) and dG (*m*/*z* 268). A 0.5 × 250 mm Zorbax SB-C18 column (5 μm in particle size; Agilent Technologies) was employed for the separation, and the flow rate was 8.0 μL/min. A 100 min gradient of 0−35% acetonitrile in 20 mM ammonium acetate was employed.

Authentic d(G[8−5]U) was employed as external standard for the LC-MS/MS quantification experiment. In this context, a series of standard solutions which contained 1.5 nmol of 2′-deoxyguanosine along with different amounts of the d(G[8−5]U) (50, 175, 350, 1000, and 1400 fmol) were submitted for LC-MS/MS analyses. Ratios of peak areas found in the selected-ion chromatograms (SICs) for monitoring the *m*/*z* 556 → 458 {for the d(G[8−5]U) cross-link} and the *m*/*z* 268 → 152 (for dG) transitions were then plotted against the molar ratios of d(G[8−5]U) over dG to give the calibration curve. The frequencies for the formation of d(G[8−5]U) per dG residue in duplex DNA and different G-quadruplexes were calculated from the calibration curve and the measured peak area ratios for the samples. These frequencies were then converted to the formation frequencies of d(G[8−5]U), i.e., the number of d(G[8−5]U) formed per ^{Br}dU, with consideration of the numbers of dG and ^{Br}dU residues present in the original ODN substrate. For instance, there are 12 dG and 1 ^{Br}dU in Tel-26-^{Br}dU$_1$ (see Results), and the ratio of d(G[8−5]U) per dG was multiplied by 12 to afford the yield of d(G[8−5]U) per ^{Br}dU reported in the paper.

RESULTS AND DISCUSSION

To explore the conformation-dependent cross-link product formation in G-quadruplex DNA, we employed 22- and 26-mer sequences derived from human telomeric DNA, i.e., 5′-d[A-(GGGTTA)$_3$GGG]-3′ (Tel22) and 5′-d[AAA(GGGTTA)$_3$GG-GAA]-3′ (Tel26), as models for the study (Figure 1b). These sequences were selected because they exist in different conformations in Na^+ or K^+ solution (Tel22 adopts a distinct basket-type structure in Na^+ solution, whereas Tel26 assumes a hybrid-type structure in K^+ solution; Figure 1a,b) as supported by NMR structural studies (*8*, *9*). The first thymidine residue in each of the three TTA loops in Tel22 and Tel26 was substituted individually with a ^{Br}dU to generate six ODN sequences. The CD spectra of ^{Br}dU-substituted and unsubstituted Tel22 are similar; both spectra exhibit a positive band at 295 nm and a negative band at 265 nm in the presence of 100 mM Na^+, which is characteristic of a basket-type G-quadruplex (Figure 2). On the other hand, when in the presence of 100 mM K^+, Tel26 with or without ^{Br}dU gives a strong positive peak at 290 nm with a shoulder peak at 268 nm, and a smaller negative peak at 240 nm, which are diagnostic of hybrid-type G-quadruplex structure (Figure 2). Thus, the replacement of thymidine residues in the loops with ^{Br}dU does not perturb the

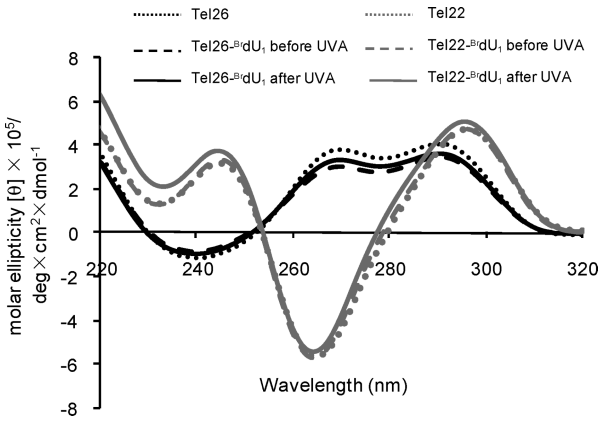

FIGURE 2: CD spectra of the ^{Br}dU-substituted and unsubstituted human telomeric strands before or after irradiation with UVA light in 100 mM Na^+ (for Tel22) or K^+ (for Tel26) solution for 1 h. Tel26-(22)-^{Br}dU$_1$ designates the strand where ^{Br}dU replaces the first thymidine residue in the first TTA loops.

folding of G-quadruplex DNA. After exposure to 0.25 J/cm^2 of UVA light, no apparent alteration in CD signal of these six ^{Br}dU-substituted strands was observed (Figure 2), suggesting that the G-quadruplex structures remained intact upon exposure to this dose of UVA irradiation.

The irradiation mixtures of ^{Br}dU-containing Tel22 and Tel26 were desalted by HPLC and digested with four enzymes (see Materials and Methods) to release the photoinduced intrastrand cross-link as a dinucleoside monophosphate, d(G^U) (*16*−*18*). The digestion mixture was subsequently analyzed by LC-MS/MS, where we assessed the formation of the d(G^U) intrastrand cross-link by monitoring the fragmentation of its $[M + H]^+$ ion (*m*/*z* 556). The 19.8 min fraction exhibits a similar retention time and identical tandem mass spectrum as the authentic d(G[8−5]U) (structure shown in Figure 1c); both reveal the formation of a dominant fragment ion at *m*/*z* 458 (Supporting Information Figure S1), which arises from the neutral loss of a 2-deoxyribose moiety. This result supports that d(G[8−5]U) is present in the enzymatic digestion mixture. It is worth noting that the cross-link was barely detectable in the digestion mixture of Tel26 or Tel22 that was not exposed with UV light. In addition, LC-MS/MS data also displayed the presence of 2′-deoxyuridine (dU) in the digestion mixture (spectrum not shown). This observation supports that, aside from the formation of the intrastrand cross-link product, the UVA light-induced dehalogenation also led to the formation of dU. This result is in line with what we found previously for the UVB-induced dehalogenation of ^{Br}dU to give dU in cellular DNA (*17*).

LC-MS/MS quantification results demonstrated that the d(G-[8−5]U) was formed efficiently (Figure 3 and Table S1 in the Supporting Information. The calibration curve is shown in Supporting Information Figure S2). In the presence of K^+ ion, the yield for d(G[8−5]U) was very similar when ^{Br}dU is situated in the first, second, or third loop of Tel26 (Figure 3). However, in the presence of Na^+ ion, the yield for d(G[8−5]U) formed from the G^{Br}U site in the second loop of Tel22 was approximately 2−3-fold higher than that formed from the corresponding site in the first or third loop (Figure 3 and Supporting Information Table S1).

Our previous study showed that $\pi-\pi$ stacking plays an important role in the sequence-dependent formation of intrastrand cross-link products in ^{Br}dU-containing duplex DNA, where the nucleobase stacking facilitates the transferring of an electron from guanine to its neighboring 5-bromouracil, thereby

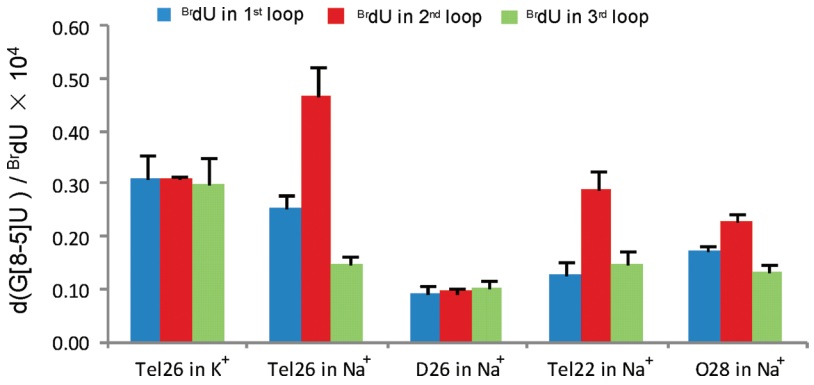

FIGURE 3: Preferential formation of d(G[8−5]U) intrastrand cross-link in the diagonal loop of G-quadruplex in Na^+ solution. Error bars represent the standard deviations for results from three independent photoirradiation and LC-MS/MS measurements.

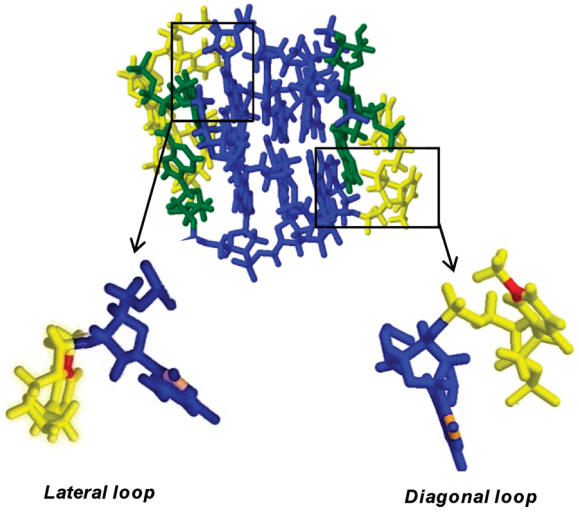

FIGURE 4: (Top) NMR structure of Tel22 in Na^+ solution. (Bottom) A close-up view of the loop region. 2′-Deoxyguanosines are in blue, C8 of guanine is in orange, and C5 of thymine is in red.

leading to the formation of G[8−5]U (*16*). In this context, NMR solution structure reveals that the first (5′) thymine in the diagonal loop of the Na^+ form of Tel22 stacks better with its neighboring 5′ guanine (Figure 4) than the corresponding thymines in the two lateral loops. Since ^{Br}dU and thymidine are isosteric, we may assume that the ^{Br}dU in the diagonal loop also stacks favorably with its vicinal 5′ guanine than the corresponding stacking of the ^{Br}dU that replaces the thymidine in either of the two lateral loops, thereby rendering more facile formation of d(G[8−5]U) from the ^{Br}dU in the diagonal loop. Moreover, a shorter distance between the C8 of guanine and the C5 of its neighboring ^{Br}dU in the diagonal loop may also assist the coupling between the two carbon atoms and enhance the formation of d(G[8−5]U) (Supporting Information Figure S3, again with the assumption that the replacement of thymidine with ^{Br}dU does not affect the structure of the G-quadruplex). Our findings are in line with previous observations made by Xu et al. (*19*), where hydrogen abstraction from the 2-deoxyribose moiety of the neighboring 5′ nucleoside in the diagonal loop was more favorable than the corresponding hydrogen abstraction in the laternal loops when the 5-iodouracil-containing G-quadruplex was exposed with UVB light (280−320 nm).

To confirm the structural requirement for the differential formation of d(G[8−5]U) in loop regions of the Na^+-form G-quadruplex, we further examined the photoreactivity of ^{Br}dU-containing ODN 5′-d[GGGG(TTTTGGGG)$_3$]-3′ (O28) in Na^+ solution (Figure 1b). This sequence is from the *Oxytricha* telomeric repeat that exists in the basket-type G-quadruplex conformation in Na^+ solution as revealed by NMR study (*20*). The yield for d(G[8−5]U) was again the highest when ^{Br}dU was situated in the diagonal loop (Figure 3). This result suggests that the unique distribution of cross-link products formed in the three loops might be useful for probing the G-quadruplex structure with a diagonal loop.

We also assessed the formation of the cross-link product in Tel26 in the presence of its complementary strand (5′-d[AAA(GGGTTA)$_3$GGGAA]-3′/5′-d[TTCCC(TAACCC)$_3$TTT]-3′, D26) and Tel26 in Na^+ solution. After annealing, D26 adopts a B-DNA structure, whereas Tel26 exists in a similar G-quadruplex conformation as Tel22 in Na^+ solution (Figure 2 and Supporting Information Figure S4) (*9*). Similar to what was found for Tel22 in Na^+ solution, we observed the highest yield for d(G[8−5]U) in the strand with ^{Br}dU being located in the second loop, suggesting that Tel26, like Tel22, adopts a G-quadruplex conformation with a diagonal loop in Na^+ solution (Figure 3). As expected, we did not observe any apparent difference in the yield for d(G[8−5]U) when ^{Br}dU is placed in different TTA trinucleotide sites in a double-stranded DNA, D26 (Figures 1b and 3). It is worth noting that the yields for d(G[8−5]U) in Tel26 with G-quadruplex conformation were at least 2-fold higher than those in double-stranded DNA, indicating that the incorporation of ^{Br}dU to the G-quadruplex motif may introduce hot spots for UVA-induced cross-link product formation.

To conclude, d(G[8−5]U) can be induced efficiently in ^{Br}dU-substituted G-quadruplex DNA upon irradiation with UVA light, with the yield being the highest when ^{Br}dU was placed in the diagonal loop of the basket-type G-quadruplex. However, the yields for the cross-link product formed in the three loops of the hybrid-type G-quadruplex were comparable. Therefore, the ^{Br}dU-mediated photo-cross-linking chemistry may serve as a useful analytical tool for monitoring the different conformations of G-quadruplex folding.

The efficient formation of intrastrand cross-link in the loop region of G-quadruplex, together with the ubiquitous presence of G-quadruplex motifs in the human genome, may bear important implications in the ^{Br}dU-induced photosensitizing effect (*13*). In this respect, it has been established that intrastrand cross-link products compromise both the efficiency and fidelity of DNA replication *in vitro* (*21*−*24*) and *in vivo* (*24*, *25*). For instance, it was found that the structurally related G[8−5]C and G[8−5 m]T, where the C8 of guanine is covalently joined with the C5 of its

adjacent 3′ cytosine and the 5-methyl carbon of its neighboring 3′ thymine, respectively, can block the progression of DNA replication mediated by purified DNA polymerases or the DNA replication machinery in cells (*21*−*24*). Moreover, the replication of these two cross-link lesions in *Escherichia coli* or mammalian cells could give rise to mutations (*24*, *25*). Furthermore, these intrastrand cross-links destabilize DNA helical structure and can be recognized by the *E. coli* UvrABC excision nuclease *in vitro* (*26*, *27*). Therefore, the incorporation of ^{Br}dU into genomic DNA and the subsequent UVA-mediated formation of the G[8−5]U intrastrand cross-link in G-quadruplex motifs in telomeric DNA and promoter regions of genes may perturb the maintenance of the integrity of the human telomere and affect the transcriptional activities of genes.

SUPPORTING INFORMATION AVAILABLE

CD spectra, LC-MS/MS results, and the distances between relevant atoms in G-quadruplexes obtained from Protein Data Bank files. This material is available free of charge via the Internet at http://pubs.acs.org.

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