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A DNA polymerase stop assay for G-quadruplex-interactive compounds

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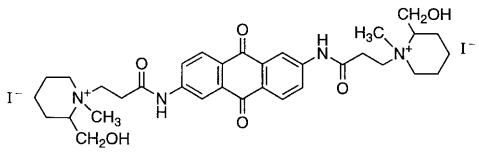
ABSTRACT

We have developed and characterized an assay for G-quadruplex-interactive compounds that makes use of the fact that G-rich DNA templates present obstacles to DNA synthesis by DNA polymerases. Using *Taq* DNA polymerase and the G-quadruplex binding 2,6-diamidoanthraquinone BSU-1051, we find that BSU-1051 leads to enhanced arrest of DNA synthesis in the presence of K^+ by stabilizing an intramolecular G-quadruplex structure formed by four repeats of either TTGGGG or TTAGGG in the template strand. The data provide additional evidence that BSU-1051 modulates telomerase activity by stabilization of telomeric G-quadruplex DNA and point to a polymerase arrest assay as a sensitive method for screening for G-quadruplex-interactive agents with potential clinical utility.

INTRODUCTION

G-rich DNA is known to assume highly stable structures formed by Hoogsteen base pairs between guanine residues (1,2). These structures, known as G-quadruplexes, are stabilized in the presence of K^+ and may have biological roles that are yet to be determined (3–5). One particular region of the genome where these structures may play a significant biological role is at the ends of chromosomes where G-rich DNA is normally found (e.g. TTAGGG and TTGGGG tandem repeats in human cells and ciliate *Tetrahymena*, respectively) (3,6,7). In addition, a number of genes containing G-rich DNA have been identified recently and it has been proposed that the G-rich regions within these genes may regulate gene expression by forming G-quadruplex structures (8–11). One potential biologically relevant role of G-quadruplex DNA is as a barrier to DNA synthesis (12). This barrier has been thoroughly investigated and has been found to be K^+ dependent (13). This observation strongly suggests that the formation of G-quadruplex species is responsible for the observed effect on DNA synthesis (14).

We have recently shown that the 2,6-diamidoanthraquinone BSU-1051 modulates human telomerase activity by a mechanism that is dependent on elongation of the telomeric primer $d(TTAGGG)_3$ to a length that is then capable of forming an intramolecular G-quadruplex structure (15). Here we show that BSU-1051, by virtue of its interaction with G-quadruplex DNA, enhances the block of DNA synthesis by the G-quadruplex structure in the presence of K^+.



BSU-1051

MATERIALS AND METHODS

DNA oligonucleotides

The DNA primer extension sequence P18 (5′-TAATACGACT-CACTATAG-3′) and the template sequences shown in Table 1 were synthesized using a PerSeptive Biosystems Expedite 8909 synthesizer and purified with denaturing polyacrylamide gels. The template DNA was diluted to 5 ng/µl and dispensed into small aliquots.

DMS methylation protection assay

The ^{32}P-labeled PQ74 and HT4 templates were denatured by heating at 90°C for 5 min and then cooled down to room temperature in 50 mM Tris–HCl buffer with or without 100 mM K^+. One microliter of 1:4 ethanol-diluted dimethylsulfate (DMS) was added to 1 µg (300 µl) of annealed DNA. Aliquots were taken at time points as indicated in the figures and modification reactions were stopped by adding 1/4 vol. of stop buffer containing 1 M β-mercaptoethanol and 1.5 M sodium acetate. The modification products were ethanol precipitated twice and treated with piperidine. After ethanol precipitation, the cleaved products were resolved on a 16% polyacrylamide gel.

DNA synthesis arrest assay

This assay is a modification of that described by Weitzmann and co-workers (14). Briefly, primers (P18, 24 nM) labeled with γ-^{32}P were mixed with template DNA (12 nM) in a Tris–HCl buffer (10 mM Tris, pH 8.0) containing K^+ (5 mM for the PQ74 template and 50 mM for the HT4 template) and denatured by

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Table 1. DNA template sequences used in this study

Name	Sequence
PQ74	5′-TCCAA CTATG TATAC **TTG^{1} G^{2} G^{3} G^{4} GTT G^{5} G^{6} G^{7} G^{8} GTT G^{9} G^{10} G^{11} G^{12} GTT G^{13} G^{14} G^{15} G^{16} GTT-** AGCGGCAC GCAAT TGCTA TAGTG AGTCG TATTA-3′
HT4	5′- TCCAA CTATG TATAC **TTA G^{1} G^{2} G^{3} TTA G^{4} G^{5} G^{6} TTA G^{7} G^{8} G^{9} TTA G^{10} G^{11} G^{12} G-** ACATATCGAT GAAAT TGCTA TAGTG AGTCG TATTA -3′
7-deaza-dG-PQ74	5′-TCCAA CTATG TATAC **TTGgGgTTGGGGTTGgGgTTGGGGTT-** AGCGGCAC GCAAT TGCTA TAGTG AGTCG TATTA-3′
7-deaza-dG-HT4	5′- TCCAA CTATG TATAC **TTAGgGTTAGGGTTAGgGTTAGGG-** ACATATCGAT GAAAT TGCTA TAGTG AGTCG TATTA -3′

G, deoxyguanine; g, 7-deaza-deoxyguanine. The G-rich telomeric sequences are shown in bold letters.

heating at 90°C for 5 min. After cooling down to room temperature, BSU-1051 was added at various concentrations and incubated at room temperature for 15 min. The primer extension reactions were initiated by adding dNTP (final concentration 100 µM), $MgCl_2$ (final concentration 3 µM) and *Taq* DNA polymerase (2.5 U/reaction; Boehringer Mannheim). For sequencing reactions, the TaqTrack Sequencing System (Promega) was used. The sequencing reaction buffer was changed to 50 mM Tris–HCl, pH 9.0, 10 mM $MgCl_2$ and 50 mM K^+. The reactions were stopped by adding an equal volume of stop buffer (95% formamide, 10 mM EDTA, 10 mM NaOH, 0.1% xylene cyanol, 0.1% bromphenol blue). For the temperature-dependent experiments, the ligand concentration was fixed and the primer extension reactions were carried out at the temperatures indicated in Figure 4. The products were separated on a 12% polyacrylamide sequencing gel. The gels were then dried and visualized on a PhosphorImager (Molecular Dynamics model 445 S1).

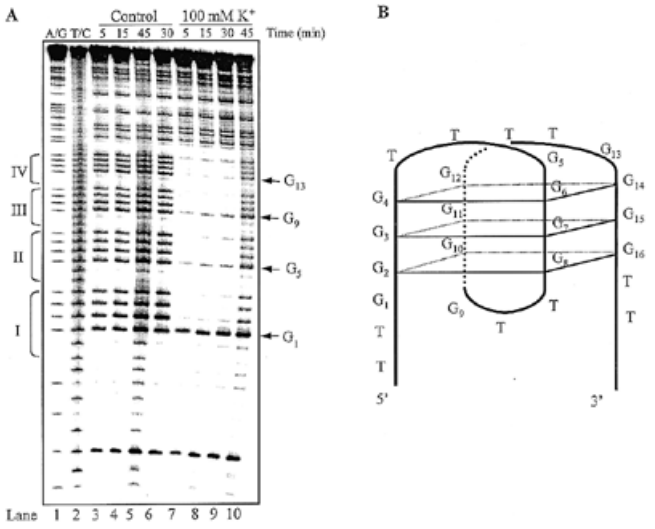

Figure 1. DMS methylation protection of the PQ74 template in K^+ buffer. (**A**) Autoradiogram of a denaturing PAGE gel showing the DMS methylation pattern in TE buffer (lanes 1–5) and 100 mM K^+ (lanes 6–10). Brackets I–IV indicate the four telomeric repeat sequences. (**B**) Proposed G-quadruplex model formed by the PQ74 template.

RESULTS

The G-rich regions of the PQ74 and HT4 templates form intermolecular G-quadruplex structures in K^+ buffer

To determine the nature of the G-quadruplex structures formed by the template sequences used in this study (Table 1), DMS was used to probe the accessibility of N7 of guanine in the DNA templates (16). As shown in Figure 1A, when the PQ74 template was methylated in 1× TE buffer, there was no apparent protection of any guanine N7. However, with the exception of the first guanine in each of the four TTGGGG repeats, all the guanines in the G-rich region of the PQ74 template are protected from reacting with DMS in 100 mM K^+ buffer, whereas guanines located outside the four repeats react strongly with DMS. This DMS protection pattern for the G-rich region of the PQ74 template in K^+ buffer suggests that only three guanines in each of the four TTGGGG repeats are involved in G-tetrad formation. This DMS reaction pattern is different from that observed previously by Henderson and co-workers (17) with the $d(TTGGGG)_4$ G-quadruplex, in which only the first guanine of the third repeat (corresponding to G9 in the PQ74 template) is hypersensitive to DMS methylation. On the basis of the results from our study, we propose a model for the G-quadruplex structure formed by the G-rich region of the PQ74 sequence consisting of $d(TTGGGG)_4$. In this model, the first guanine of the first repeat (G1 in Table 1 and Fig. 1B) is located in the 5′-overhang region and is therefore open to DMS methylation. However, the first guanines of the second, third and fourth repeats (G5, G9 and G13, respectively) are located in the loop regions of the G-quadruplex. Although the N7 groups of these three loop guanines are not involved in hydrogen bonds, steric inaccessibility may protect them from DMS methylation. The DMS footprinting pattern shows that while they are partially protected from DMS methylation, this protection is less than that for the other guanines in the repeat.

The TTAGGG repeats in the G-rich region of the HT4 template also showed high DMS methylation protection in K^+ buffer (data not shown). In this particular case, all three guanines in each

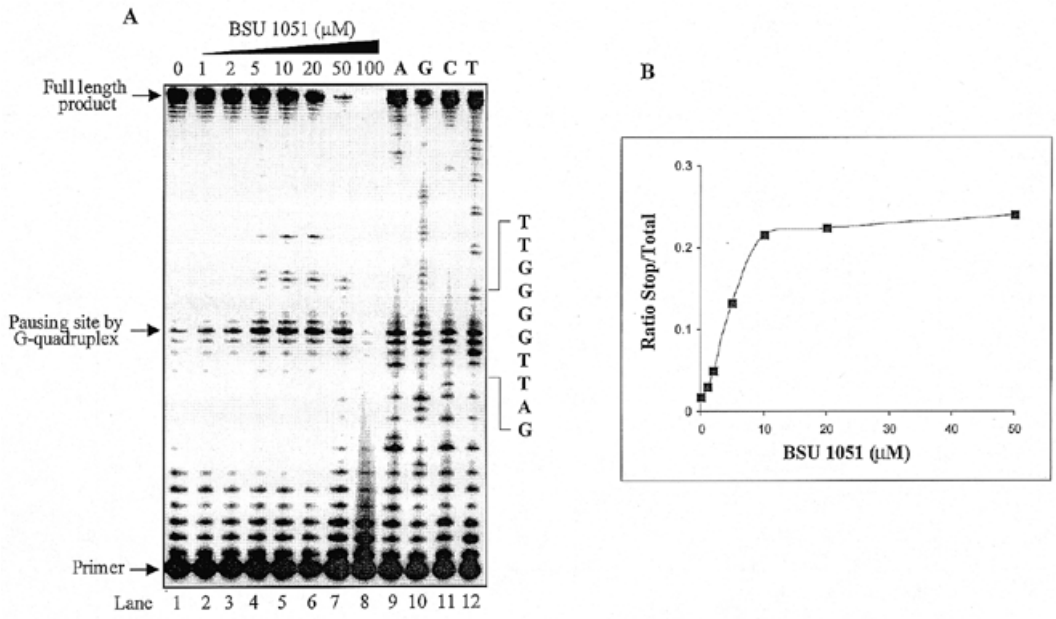

Figure 2. BSU-1051 concentration-dependent block of *Taq* polymerase DNA synthesis by the G-quadruplex structure formed on the PQ74 template at 55°C. (**A**) Autoradiogram of a sequencing gel showing enhanced DNA synthesis pausing at the G-quadruplex site with increasing concentrations of BSU-1051 (lanes 1–8). Arrows indicate the positions of the full-length product of DNA synthesis, the G-quadruplex pausing site and the free primer. (**B**) Quantitation of the gel using ImageQuant software. The *y*-axis shows the ratio of radiation volume of pausing sites to total radiation volume of the lane. The *Taq* polymerase activity is normalized by the total primer extension products, including premature products, G-quadruplex pausing products and full-length products.

repeat were almost evenly protected from methylation, indicating that all of them are involved in G-tetrad formation. This DMS methylation pattern is consistent with the intramolecular G-quadruplex structure proposed by Patel and co-workers for the d[$AG_3(T_2AG_3)_3$] sequence based on NMR studies (18).

BSU-1051 binds to G-quadruplex DNA and blocks DNA synthesis in a concentration-dependent manner

Although it has been shown that G-quadruplex structures block primer extension by DNA polymerase in a K^+-dependent manner (14), we are unaware of any reports showing enhanced blockage by G-quadruplex-interactive agents. To determine if BSU-1051 binding to G-quadruplex enhances the block to DNA synthesis, primer extension reactions were carried out in the absence and presence of BSU-1051. Figures 2A and 3A show the results of *Taq* DNA polymerase primer extension on DNA templates containing four repeats of either TTGGGG (PQ74, Fig. 2A) or TTAGGG (HT4, Fig. 3A) in the presence of different concentrations of BSU-1051 at 55°C. In these experiments, K^+ was added at low concentrations (5 mM K^+ for the PQ74 template and 20 mM K^+ for the HT4 template) in order to prevent overwhelming polymerase pausing due to formation of highly stable G-quadruplex structures. In the absence of BSU-1051, there is only a slight pausing of the *Taq* DNA polymerase when it reaches the 3′-end of the G-rich site on the template DNA at 55°C (Figs 2A and 3A, lanes 1). However, upon increasing the concentration of BSU-1051, enhanced pausing is observed at the same site as that seen with low K^+ concentrations. This suggests that BSU-1051 enhances the polymerase pausing by stabilizing the G-quadruplex structure formed in the K^+ buffer. At high BSU-1051 concentrations, we not only observed enhanced pausing at the 3′-end of the G-quadruplex site but also increased premature termination resulting from non-specific interactions between BSU-1051 and the single-stranded template DNA (Figs 2A and 3A, lanes 7 and 8). At a BSU-1051 concentration of 100 μM, primer extension is completely inhibited due presumably to non-specific interactions between BSU-1051 and the single- and/or double-stranded DNA or between BSU-1051 and the polymerase itself. In addition to the primary pausing site at the beginning of the G-quadruplex site, two other secondary pausing sites at the second and third G-rich repeats are observed at high BSU-1051 concentrations. These pausings are probably induced by other structures formed by this G-rich sequence. Given the fact that secondary pausing beyond the first G-tetrad is not seen in the sequencing lanes that contain 50 mM K^+, it is likely that these secondary pausings are caused by hairpin structures that are stabilized by BSU-1051 but not K^+. This suggests that BSU-1051 has a relatively higher affinity for G-quadruplex DNA over other DNA secondary structures or single- and double-stranded DNA.

DNA synthesis arrest by the BSU-1051–G-quadruplex complex depends on the stability of the G-quadruplex structure

To further evaluate the ability of BSU-1051 to stabilize G-quadruplex DNA, *Taq* DNA polymerase primer extension reactions were carried out at five different temperatures in the presence and absence of BSU-1051. As shown in Figure 4A, in the absence of BSU-1051 polymerase pausing on the PQ74 template containing four repeats of TTGGGG is almost lost at ~65°C (lane 4), which is presumably the melting point of the G-quadruplex structure formed by this G-rich region in the template DNA. On the other hand, in the presence of 20 μM BSU-1051, the G-quadruplex structure is further stabilized and significant pausing is observed up to 74°C. In the HT4 template containing four repeats of TTAGGG, in which the G-quadruplex structure formed is presumably less stable, pausing fades out at

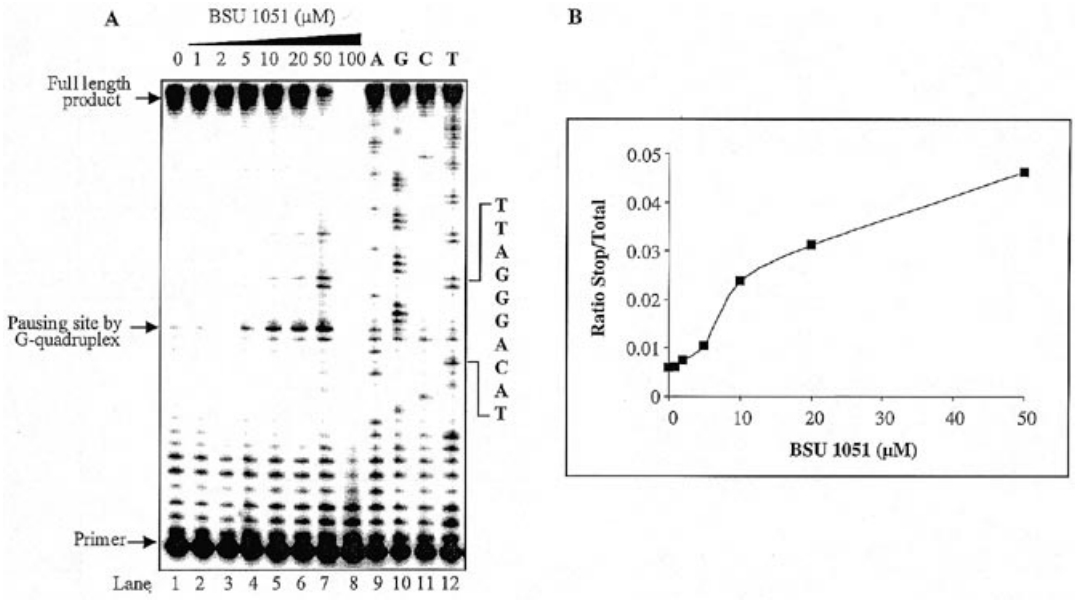

Figure 3. BSU-1051 concentration-dependent block of *Taq* polymerase DNA synthesis by the G-quadruplex structure formed on the HT4 template at 55°C. (**A**) Autoradiogram of a sequencing gel showing enhanced DNA synthesis pausing at the G-quadruplex site with increasing concentrations of BSU-1051 (lanes 1–8). Arrows indicate the positions of the full-length product of DNA synthesis, the G-quadruplex pausing site and the free primer. (**B**) Quantitation of the gel using ImageQuant software is the same as in Figure 2B.

55°C in the absence of the ligand (Fig. 4B, lane 3). However, in the presence of BSU-1051, pausing is observed up to 65°C (Fig. 4B, lane 9). Thus, for both DNA sequences, ΔT_m upon addition of 20 μM BSU-1051 is ~20°C.

In order to confirm that the pausings seen in Figures 2A and 3A result from the formation of a G-quadruplex structure on the template DNA, certain guanines in the templates were substituted with 7-deaza-dG. Since N7 of guanine is involved in hydrogen bonding in the formation of a G-quadruplex structure, substitution of guanine with 7-deaza-dG should preclude the formation of any G-quadruplex structure and allow for uninterrupted primer extension on the template by *Taq* DNA polymerase in the presence of either K^+ or BSU-1051. As shown in Table 1, two guanines in the TTAGGG repeat region of the HT4 template and four guanines in the TTGGGG repeat region of the PQ74 template were replaced with 7-deaza-dG. This change would allow the formation of no more than two intramolecular G-tetrads and should lead to destabilization of the intramolecular G-quadruplex structure. The primer extension results with these 7-deaza-dG substituted templates indicate that no significant pausing occurs in either template in the presence of up to 20 mM K^+ or at BSU-1051 concentrations of up to 50 μM (data not shown). This result provides strong support for the conclusion that BSU-1051 binds to and stabilizes intramolecular G-quadruplex DNA, leading to pronounced DNA synthesis arrest at the G-quadruplex site in the original G-rich templates.

DISCUSSION

G-rich sequences such as telomeric DNA and triplet DNA have been reported to form parallel or antiparallel G-quadruplex structures in the presence of monovalent cations such as Na^+ and K^+. Williamson and co-workers observed very strong intramolecular UV crosslinking for the sequence $d(TTGGGG)_4$ in a 50 mM K^+ buffer (5). Their results indicate that this sequence forms an intramolecular structure. Using DMS methylation, we conlcude that four repeats of TTGGGG or TTAGGG within a non-G-rich sequence are capable of forming an intramolecular G-quadruplex structure in K^+ buffer. Furthermore, the DMS methylation results indicate that of the possible types of G-quadruplex structures that could be formed by $d(TTGGGG)_4$, a structure consisting of three G-tetrads is the predominant species in 100 mM K^+ buffer. The proposed G-quadruplex structure formed by $d(TTGGGG)_4$ shown in Figure 1B has a diagonal loop, but an alternative intramolecular G-quadruplex structure formed by foldover hairpins consisting of three G-tetrads is also possible (1,19,20). However, we could not differentiate between these two different types of intramolecular G-quadruplex structures by the DMS methylation pattern alone.

G-rich sequences that are capable of forming G-quadruplexes *in vitro* can be found in telomeric sequences (21–23), immunoglobulin switch regions (8), the insulin gene (9), the control region of the retinoblastoma susceptibility gene (10), the promoter region of the c-*myc* gene (11), fragile X syndrome triplet repeats (2,24) and HIV-1 RNA (25). Although direct evidence for the existence of G-quadruplexes *in vivo* is lacking, the ability of these important sequences to form G-quadruplex structures *in vitro* suggests that G-quadruplex DNA may play important roles in several biological events. For instance, it has been suggested by Sen and Gilbert that telomeric DNA sequences may associate to initiate the alignment of four sister chromatids by forming parallel guanine quadruplexes (26). Furthermore, the discovery of G-quadruplex-forming sequences in the promoter region of certain genes suggests that G-quadruplex structures may play a role in the transcription regulation of these genes. Another possible role of G-quadruplex DNA is the regulation of telomere length, since a telomeric overhang that forms a G-quadruplex structure would not be a good substrate for telomerase (27,28). We have recently demonstrated that

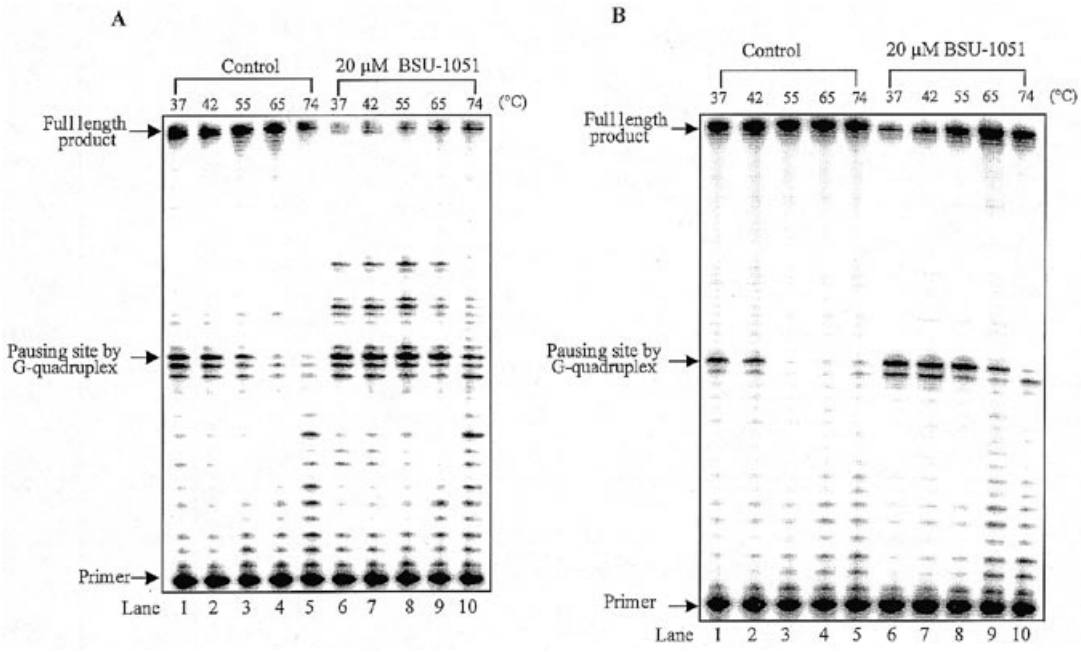

Figure 4. Temperature-dependent block of DNA synthesis by the G-quadruplex structure formed on the PQ74 and HT4 templates in the absence or presence of BSU-1051. (**A**) Autoradiogram of a sequencing gel showing enhanced temperature resistance of G-quadruplex pausings in the presence of 20 μM BSU-1051 (compare lanes 1–5 with lanes 6–10). Arrows indicate the positions of the full-length product of DNA synthesis, the G-quadruplex pausing site and the free primer. (**B**) Autoradiogram of a sequencing gel showing G-quadruplex pausings at higher temperatures in the presence of 20 μM BSU-1051. Arrows indicate the positions of the full-length product of DNA synthesis, the G-quadruplex pausing site and the free primer.

BSU-1051 inhibits primer extension by telomerase only when the substrate (telomeric DNA) reaches four or more repeats in length (15). In this report, we show that BSU-1051 is able to bind to and stabilize the intramolecular G-quadruplex structure formed by four telomeric repeats. Thus, it is reasonable to postulate that BSU-1051 inhibits telomerase by interacting with its substrate (G-quadruplex-forming telomeric repeats) rather than telomerase itself. If G-quadruplex structures play important roles in other biological processes, then G-quadruplex-interactive compounds such as those described here, which stabilize these structures, may have a variety of biological effects. A series of 2,6-diamidoanthraquinones, including BSU-1051, has been reported to moderate conventional cytotoxicity in a range of tumor cells (29,30) and to inhibit human telomerase (31). The G-quadruplex binding property of those compounds provides a possible mechanism for their action, although other mechanisms involving targeting of duplex DNA are also likely.

Despite the lack of unequivocal evidence for the structure of the BSU-1051–G-quadruplex complex, we have recently proposed a model for a perylene–G-quadruplex complex based on NMR evidence (32). By analogy with this structure and that proposed for a $TMPyP_4$–G-quadruplex structure (33), it seems most likely that the binding site of BSU-1051 is external to the lower G-tetrad and within the diagonal loop.

The block of DNA synthesis by G-quadruplex structures is not polymerase specific. Woodford and co-workers showed that the K^+-dependent DNA synthesis arrest by G-quadruplex structures is similar for various polymerases (13). We have found that the BSU-1051-induced DNA synthesis arrest pattern is virtually identical when *Taq* DNA polymerase, *Escherichia coli* DNA polymerase I (Klenow fragment) or AMV reverse transcriptase is used (data not shown). Given the fact that many G-rich DNA sequences are capable of forming G-quadruplexes *in vitro* (particularly some cancer-related genes and sequences such as c-*myc* and telomeres), G-quadruplex promises to be a potential target for anticancer chemotherapy. The DNA synthesis stop assay described in this report provides a simple and rapid method for the identification of G-quadruplex-interactive agents as potential lead compounds. This polymerase stop assay also allows an internal comparison for the relative binding of potential G-quadruplex-interactive compounds with single- and double-stranded DNA targets. This is an important comparison that may provide clues as to the relative cytotoxicity of these compounds.

We have successfully used the present assay in the identification and characterization of other G-quadruplex-interactive compounds that are also telomerase inhibitors (32,33). However, since not all telomerase inhibitors are G-quadruplex-interactive compounds, the utility of the present assay is not primarily as a screen for telomerase inhibitors and it is not meant to substitute for a direct assay of telomerase inhibition. Rather, this assay can be used to identify other G-quadruplex-interactive compounds with potential clinical utility or of practical use in the study of transcription, recombination, generic disorders and other biological processes where G-quadruplex structures may be involved.

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