# Inhibition of Unwinding of G-Quadruplex Structures by Sgs1 Helicase in the Presence of *N*,*N*′-Bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic Diimide, a G-Quadruplex-Interactive Ligand†

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ABSTRACT: *N*,*N*′-Bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER), a perylene derivative, is a very potent and selective G-quadruplex DNA-interactive agent. It has been shown to inhibit DNA polymerase and telomerase by stacking externally to the G-tetrads in the G-quadruplex structures. Recently, we have demonstrated that this small molecule greatly accelerates the assembly of G-quadruplex structures in a cell-free system. In this report, we present data demonstrating that PIPER prevents the unwinding of G-quadruplex structures by yeast Sgs1 helicase. Sgs1 belongs to the RecQ DNA helicase family whose members include other G-quadruplex DNA unwinding helicases, such as human Bloom's syndrome and human Werner's syndrome helicases. PIPER specifically prevents the unwinding of G-quadruplex DNA but not duplex DNA by Sgs1. Competition experiments indicate that this inhibitory activity is due to the interaction of PIPER with G-quadruplex structures rather than the helicase itself. These results combined with previous studies suggest a possible mechanism of action for these G-quadruplex-interactive agents inside cells: they might induce G-quadruplex formation in G-rich regions on genomic DNA, stabilize these structures, and prevent them from being cleared by enzymes such as helicases. The G-quadruplex structures may, in turn, disrupt some critical cellular events such as DNA replication, transcription regulation, and telomere maintenance.

Guanine-rich DNA sequences such as telomeric repeats (*1*, *2*), fragile X-syndrome triplet repeats (*3*), and the promoter region of the c-*myc* gene (*4*) have been shown to assume highly stable G-quadruplex structures. Although the existence of such structures in vivo remains uncertain, cellular events such as chromosomal alignment (*5*, *6*), replication (*7*), transcription regulation (*4*), and recombination (*8*, *9*) have been suggested to involve G-quadruplex structure assembly and disassembly. One piece of indirect evidence supporting this hypothesis comes from the identification of G-quadruplex structure binding proteins. Three such proteins, yeast proteins RAP1 (*10*), Hop1p (*11*), and the $\beta$-subunit of *Oxytricha* telomere binding protein (*12*), have been shown to facilitate the assembly of G-quadruplex structures and are hence called G-quadruplex structure chaperones (*13*). Recently, several helicases have been identified to unwind G-quadruplex DNA in vitro. These helicases include the viral SV40 large T-antigen helicase (*14*), the *Saccharomyces cerevisiae* Sgs1 helicase (*7*), human Werner's syndrome (WRN)[1] helicase, and Bloom's syndrome (BLM) helicase (*15*, *16*). This finding further supports the postulation that G-quadruplex structures may play important roles inside cells. These helicases not only have the G-quadruplex DNA-unwinding activity but also possess duplex DNA binding activity, duplex DNA unwinding activity, and ATPase activity (*17*). Most intriguingly, some of these helicases, such as BLM helicase and yeast Sgs1, preferentially unwind G-quadruplex DNA over duplex DNA (*7*, *16*). The Sgs1 helicase was shown to unwind G-quadruplex DNA at least 10-fold more efficiently than duplex DNA (*7*).

Sgs1 belongs to the RecQ DNA helicase family whose members include human BLM (*18*, *19*) and WRN helicases (*20*, *21*). Its G-quadruplex DNA unwinding activity is ATP- and $Mg^{2+}$-dependent (*7*). Yeast cells deficient in Sgs1 usually show increased genomic instability, including a greatly elevated level of mitotic recombination and chromosome missegregation (*22*−*24*). These cells share the same characteristic of hyperrecombination as mammalian cells deficient in BLM helicase or WRN helicase (*25*−*27*), which also unwinds G-quadruplex DNA. Moreover, the premature aging

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[1] Abbreviations: G4, G quadruplex; WRN, Werner's syndrome; BLM, Bloom's syndrome; PIPER, *N*,*N*′-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide; DMS, dimethyl sulfate; ss, single-stranded.

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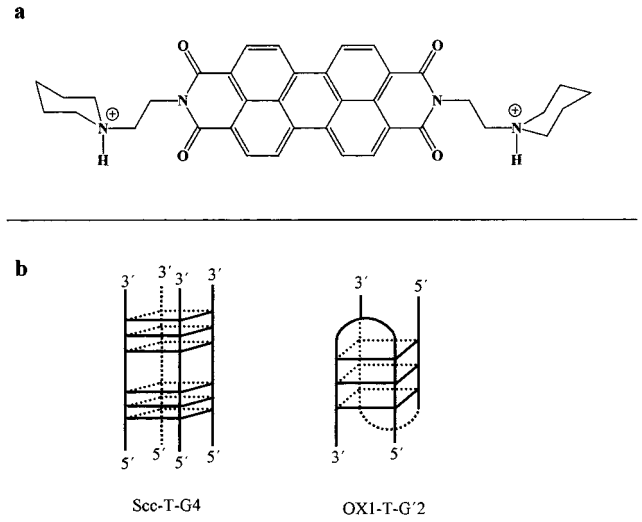
Chart 1




characteristic of Sgs1-deficient yeast is thought to be an analogy to the premature aging exhibited by Werner's syndrome patients (*22*).

G-Quadruplex-interactive compounds such as perylenes and porphyrins have been shown not only to bind to G-quadruplex structures but also to selectively facilitate the formation of certain types of G-quadruplex structures (*28*−*33*). They inhibit DNA polymerase and telomerase by interacting with G-quadruplex structures formed from the DNA sequences that are substrates for the enzymes (*30*, *32*, *34*). Here we show that the perylene analogue *N*,*N*′-bis[2-(1-piperidino)ethyl]-3,4,9,10-perylenetetracarboxylic diimide (PIPER, Chart 1a) specifically prevents the unwinding of G-quadruplex DNA by Sgs1 helicase. These results have potentially important implications in how G-quadruplex-interactive compounds may produce the biological consequences that are observed in cells (e.g., anaphase bridges and inhibition tumor cell growth) (*29*, *35*).

## EXPERIMENTAL PROCEDURES

*Helicase, PIPER, and Oligonucleotide Preparation*. A recombinant Sgs1 fragment (amino acids 400−1268 of the 1447 amino acid full-length protein) was overexpressed in yeast and purified as described previously (*17*). PIPER was synthesized and purified as described previously (*30*). DNA oligomers $H_1$, 5′-TTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTGCTAGTTGGGAAGCCGATGC-3′; $K_1$, 3′-GCATCGGCTTCCCAACTAGCTTTTTTTTTT-5′; OX1-T, 5′-ACTGTCGTACTTGATATTTTGGGGTTTTGGGGAATGTGA-3′; and Scc-T, 5′-AACTTGTGTGGGTGTGTGTGGGTGTGTGT-3′ (*7*) were synthesized on an Expedite synthesizer (PerSeptive Biosystems, Model 8909) and purified by denaturing polyacrylamide gel electrophoresis (PAGE).

*G-Quadruplex DNA Formation*. The formation of G-quadruplex DNA was carried out as described previously (*7*, *36*). Briefly, 260 $\mu$M of Scc-T or OX1-T was denatured in 1× TE containing 1 M NaCl or 1 M KCl by heating at 95 °C for 10 min. The denatured DNA was then annealed at 37 °C for 48 h. The annealed products were separated on 8% native PAGE containing 10 mM KCl and running at 4 °C for 12 h with constant current of 20 mA. Bands corresponding to tetrameric G-quadruplex, dimeric G-quadruplex, and monomeric Scc-T or OX1-T were excised from gel and eluted with 1× TE (pH 8.0) containing 50 mM NaCl and 20 mM KCl. The purified G-quadruplex DNA was 5′-$^{32}$P-labeled and purified by native PAGE. Labeled G-quadruplex DNA was precipitated with ethanol and stored in 10 mM Tris-HCl (pH 7.5) containing 100 mM KCl at −20 °C. Duplex H1/K1 was formed by annealing H1 and K1 at room temperature for 16 h and purified by native PAGE.

*Helicase Assay*. The helicase unwinding assay was performed as described previously (*7*, *17*) but with some modifications. About 10 000 cpm-labeled DNA (∼100 nM) was incubated with PIPER at concentrations indicated in Figures 1−4 in a helicase reaction buffer [50 mM Tris-HCl (pH 7.5) 2 mM $MgCl_2$, 2 mM ATP, 50 mM NaCl, and 100 $\mu$g/mL BSA] for 30 min at room temperature. Sgs1 (25 nM) was then added to the reactions, which were then incubated at 30 °C for 30 min. For competition reactions, competitor DNA was added up to the final concentrations indicated in the figure legends and incubated for 10 min before Sgs1 was added. Reactions were stopped by adding SDS and proteinase K to attain the final concentrations of 0.5% and 0.5 mg/mL, respectively, and then incubated at 37 °C for 10 min. Samples were then separated on an 8% native polyacrylamide gel containing 10 mM KCl. Gels were dried on a dryer and visualized on a PhosphorImager (Molecular Dynamics, Model 445 SI).

## RESULTS

*G-Quadruplex DNA Formation and Characterization*. Formation of G-quadruplex structures by yeast telomeric sequence Scc-T and the *Oxytricha* telomeric sequence OX1-T has been characterized and their unwinding by yeast Sgs1 helicase has been described by Sun et al. (*7*, *16*). All three oligomers can form either dimeric or tetrameric G-quadruplexes. We separated and purified these different types of G-quadruplexes using native PAGE and verified the formation of G-quadruplexes by dimethyl sulfate (DMS) footprinting. The results of DMS methylation protection are consistent with that reported by Sun et al. (data not shown) (*7*). In the subsequent helicase unwinding assays we used the tetrameric G-quadruplexes formed from Scc-T (Scc-T-G4, Chart 1b) as an example of parallel G-quadruplex unwinding by Sgs1 and dimeric G-quadruplexes formed from OX1-T (OX1-T-G′2, Chart 1b) as an example of antiparallel hairpin G-quadruplex unwinding.

*PIPER Prevents the Unwinding of both Parallel Tetrameric and Antiparallel Dimeric G-Quadruplex Structures*. We have previously demonstrated that PIPER (Scheme 1a), a perylene analogue, interacts with G-quadruplex DNA very strongly (*28*, *30*). Here we studied the effect of PIPER on the Sgs1 helicase-catalyzed unwinding of G-quadruplex structures formed from Scc-T and OX1-T. Figure 1 shows the unwinding of the parallel quadruplex formed from the yeast telomeric sequence Scc-T (Scc-T-G4) by Sgs1 helicase in the presence of PIPER. At 50 nM PIPER, which is about twice the quadruplex concentration (∼25 nM), more than 90% of the quadruplex DNA is prevented from unwinding (Figure 1 panel A, lane 3, and panel B). When the PIPER concentration reaches 0.2 $\mu$M, almost all the DNA molecules keep forming the G-quadruplex structure (Figure 1B). At high

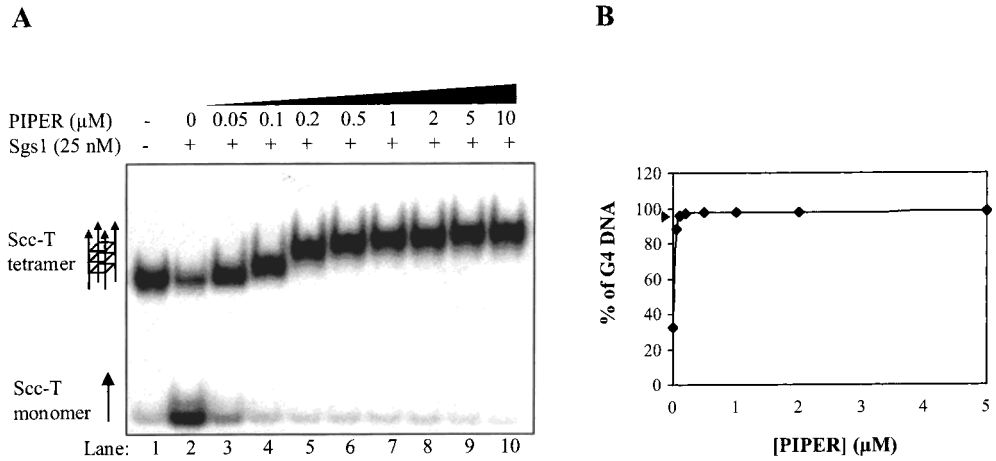



FIGURE 1: Effect of PIPER on the unwinding of parallel G-quadruplex DNA by Sgs1. (A) Lane 1, control without Sgs1 and PIPER; lanes 2−10, unwinding of Scc-T-G4 by 25 nM Sgs1 in the presence of 0, 0.05, 0.1, 0.2, 0.5, 1, 2, 5, and 10 $\mu$M PIPER. (B) Quantification of the percentage of G-quadruplex DNA by ImageQuant software (Molecular Dynamics). The arrowhead indicates the percentage of G-quadruplex DNA present in the absence of both PIPER and Sgs1. The percentage of G-quadruplex DNA in each lane was determined by the following formula: (intensity of the G-quadruplex DNA band/intensity of the total DNA) × 100.

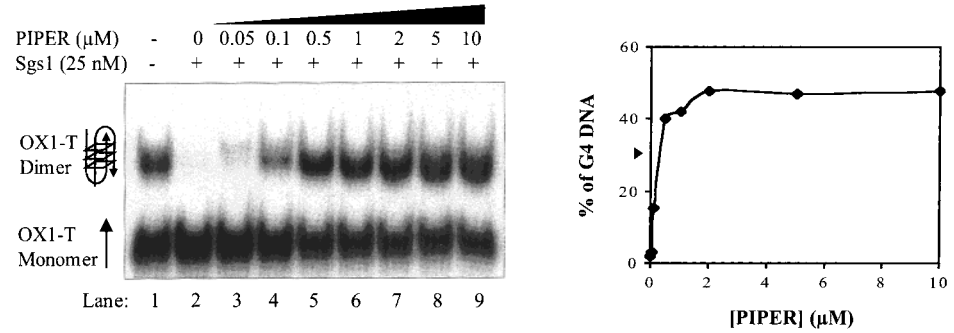



FIGURE 2: Effect of PIPER on the unwinding of dimeric antiparallel G-quadruplex DNA by Sgs1. (A) About 100 nM dimeric OX1-T-G′2 was incubated with 25 nM Sgs1 in the presence of increasing amounts of PIPER (lanes 2−9). Lane 1 is the negative control without Sgs1 and PIPER. (B) Quantification of the percentage of G-quadruplex DNA is the same as in Figure 1B. The arrowhead indicates the percentage of G-quadruplex DNA present in the absence of both PIPER and Sgs1.

PIPER concentrations the G-quadruplex band is shifted because of the binding of PIPER molecules to the G-quadruplex. The binding is saturated at about 1 $\mu$M PIPER (Figure 1A). This result indicates that PIPER binds to the parallel G-quadruplex structure with very high affinity and that the PIPER−quadruplex complex is not a substrate for Sgs1 helicase.

PIPER also prevents the unwinding of the antiparallel dimeric G-quadruplex formed from OX1-T (OX1-T-G′2). At a concentration of 0.5 $\mu$M PIPER, there is no difference in the amount of G-quadruplex DNA between the control (Figure 2A, lane 1; neither PIPER nor Sgs1 was added) and the PIPER−G-quadruplex complex, indicating complete inhibition of unwinding of OX1-T-G′2 by Sgs1. Higher concentrations of PIPER keep more OX1-T-G′2 from melting during the reaction (Figure 2A, compare lanes 6−9 with lane 1). However, even at 10 $\mu$M PIPER, only 45% of the OXI-T DNA molecules form dimeric G-quadruplexes, whereas in the case of Scc-T sequence, 100% of the DNA molecules form parallel quadruplex at a PIPER concentration of 0.2 $\mu$M. This indicates that the Scc-T tetrameric G-quadruplex is much more stable than the OX1-T dimeric G-quadruplex. One interesting observation is that PIPER does not cause OX1-T-G′2 to shift, as seen in Scc-T-G4 (compare Figures 1A and 2A). One possible reason is that PIPER molecules act as drivers in the OX1-T G-quadruplex formation, as we observed with other G-rich sequences (*28*), but do not bind strongly enough to the assembled quadruplexes to survive the 37 °C incubation and electrophoresis.

*PIPER Does Not Prevent the Unwinding of Duplex DNA by Sgs1*. Sgs1 has been reported to unwind G-quadruplex DNA at least 10 times more efficiently than it unwinds duplex DNA (*7*). To compare the effects of PIPER on Sgs1 unwinding of G-quadruplex DNA and duplex DNA, we made a forked Watson−Crick duplex H1/K1, which is a substrate of Sgs1 and other RecQ family helicases (*7*, *16*). As shown in Figure 3, even 20 $\mu$M PIPER has no effect on the Sgs1 unwinding of the H1/K1 duplex. This result indicates that PIPER has little interaction with duplex DNA, which is consistent with our observations of NMR experiments (data not shown). This result also demonstrates that PIPER does not directly inhibit the helicase activity of Sgs1 by interacting with the protein, and thus the prevention of G-quadruplex DNA unwinding by Sgs1 results from the interaction between PIPER and G-quadruplex structures.

*Inhibition of Sgs1 Activity Is Due to PIPER Specifically Interacting with G-Quadruplex DNA Rather Than Promoting*

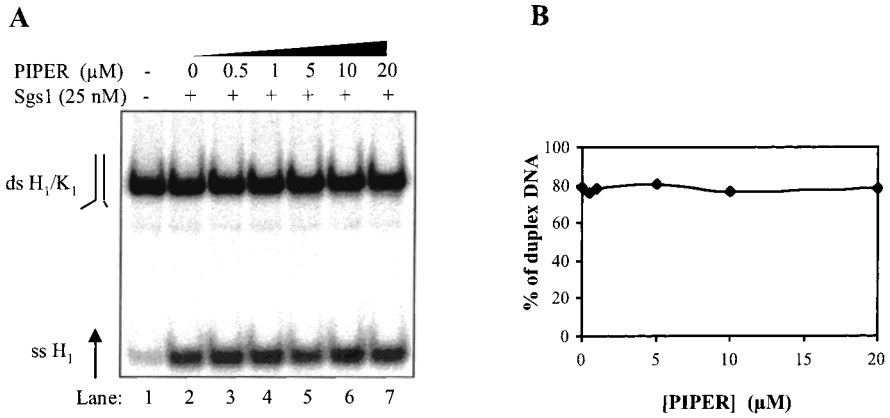



FIGURE 3: Effect of PIPER on the unwinding of forked duplex DNA $H_1/K_1$ by Sgs1. (A) Lane 1, control without Sgs1 and PIPER; lanes 2−7, unwinding of ds H1/K1 by 25 nM Sgs1 in the presence of 0, 0.5, 1, 5, 10, and 20 $\mu$M PIPER. (B) Quantification of the percentage of duplex DNA is the same as in Figure 1B.

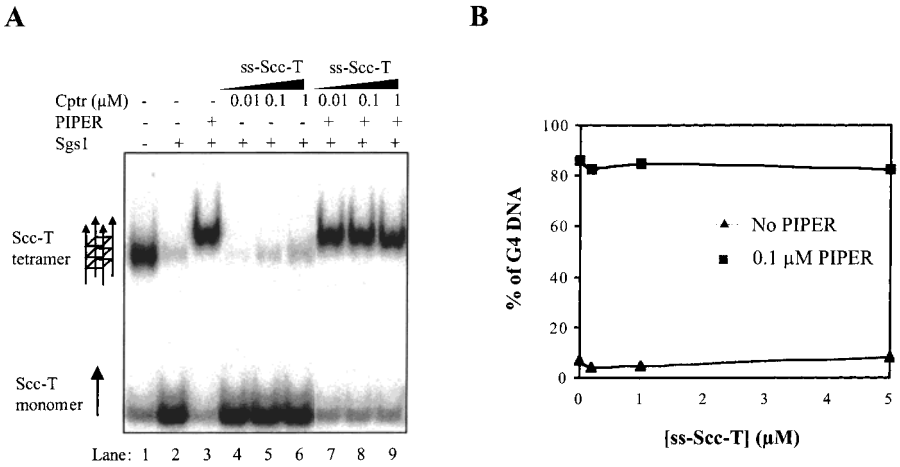


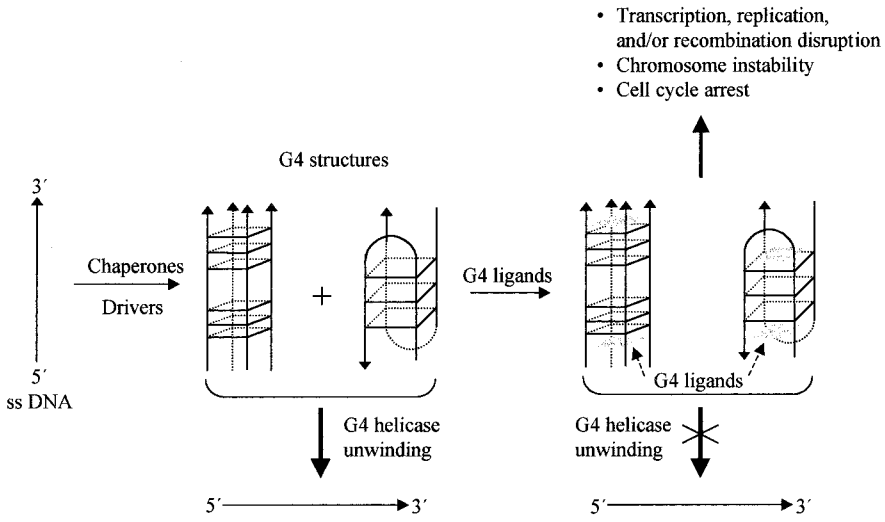

FIGURE 4: Effect of unlabeled single-stranded Scc-T (ss-Scc-T) on the unwinding of parallel G-quadruplex DNA by Sgs1 (25 nM) in the presence and absence of PIPER. (A) Unwinding of $^{32}$P-labeled parallel G-quadruplex DNA formed from Scc-T in the absence (lanes 4−6) and presence (lanes 7−9) of 0.1 $\mu$M PIPER. Lane 1, control without Sgs1, PIPER, and Scc-T. Lane 2, control without PIPER and ss-Scc-T. Lane 3, control without ss-Scc-T. Lanes 4−9, unlabeled ss-Scc-T as competitor. (B) Quantification of the gel data shown in panel A using ImageQuant software. (▲) ss-Scc-T competition in the absence of PIPER; (■), ss-Scc-T competition in the presence of PIPER.

*the Refolding of the Unwound Single-Stranded DNA.* We have previously shown that PIPER can promote the assembly of single-stranded G-rich DNA into G-quadruplex DNA (*28*). To investigate whether the prevention of G-quadruplex DNA unwinding by Sgs1 really results from G-quadruplex DNA stabilization by PIPER or whether this is an apparent effect due to the accelerated reassembly of G-quadruplex structures by PIPER, we performed a competition experiment. As shown in Figure 4, two sets of reactions, each of which involves competition between excess nonradioactive single-stranded Scc-T (ss-Scc-T) and radio-labeled Scc-T-G4, were carried out. The first set of reactions was carried out in the absence of PIPER (lanes 4−6) and the second set of reactions was carried out in the presence of 0.1 $\mu$M PIPER (lanes 7−9). In the absence of PIPER, ss-Scc-T had little effect on the Sgs1 unwinding activity (Figure 4A, lanes 4−6). In the presence of PIPER, the single-stranded Scc-T showed virtually no effect on the prevention of Scc-T-G4 unwinding (Figure 4A; compare lanes 7−9 with lane 3). These results establish that the binding of PIPER to preformed G-quadruplex DNA is the primary reason for the observed inhibition of Sgs1 unwinding of the G-quadruplex DNA and that the promotion role of PIPER is not an important contributing factor.

## DISCUSSION

Helicases that unwind duplex DNA are considered to be essential for DNA replication, repair, transcription, and recombination (*37*−*40*). The G-quadruplex DNA unwinding activity of some helicases, such as SV40 large T-antigen (*14*), yeast Sgs1 (*7*), and WRN and BLM helicases (*15*, *16*), suggests another possible role of DNA helicases: the unwinding of alternative DNA structures that are similar to G-quadruplex structures. G-quadruplex structures may arise from any biological process involving the separation of the duplex DNA. If not cleared in a timely manner, these structures may cause pausing during DNA replication, recombination, transcription, and chromosomal segregation. The clearance of these structures may need the help of other cellular proteins such as topoisomerase (*24*, *41*, *42*). In this report we demonstrate that G-quadruplex-interactive compounds can specifically prevent the unwinding of G-quadruplex DNA by Sgs1 helicase. This finding, alongside our previous results, establishes a new way of targeting DNA: inducing and stabilizing secondary structures in DNA and then preventing them from being disassembled. The secondary structures, in turn, disrupt certain critical cellular functions such telomere maintenance and transcription

Scheme 1




regulation (Scheme 1). Although this kind of strategy has not been proven in vivo, our cell-free data suggest that it might be feasible. G-Quadruplex-interactive agents such as porphyrins and PIPER have been shown to facilitate the formation of parallel G-quadruplexes from single-stranded DNA (*28*) (H. Han et al., manuscript submitted) and double-stranded DNA (A. Rangan and L. H. Hurley, unpublished data). Biochemical studies demonstrated that these compounds can inhibit telomerase and DNA polymerase by interacting with the G-quadruplex structures formed within their DNA substrates (*30*, *32*). These compounds also slow the growth of tumor cells (*29*) and induce the formation of anaphase bridges (*35*), an observation that is similar to the chromosomal aberrations observed in yeast cells with SGS1 mutations (*23*). The specificity of such compounds resides in their selectivity in inducing and stabilizing *different* types of G-quadruplexes. The design and synthesis of G-quadruplex-interactive compounds that achieve this type of selectivity in interactions with G-quadruplex structures are important objectives.

The assembly of G-quadruplex structures from single-stranded DNA is usually a very slow process (*43*, *44*). We have reported that small organic molecules such as PIPER can accelerate the assembly of G-quadruplex DNA (*28*). At first glance, it might seem possible that the acceleration of G-quadruplex DNA formation by PIPER would simulate the inhibition of G-quadruplex DNA unwinding by Sgs1; however, the competition experiments indicated that the prevention of Sgs1 unwinding of G-quadruplex DNA by PIPER is primarily a result of stabilization of G-quadruplex DNA by bound PIPER molecules, rather than the accelerated reassembly of unwound G-quadruplex DNA. This result is expected because (1) even in the presence of PIPER the assembly process is still relatively slow (the estimated rate constant for dimeric quadruplex assembly is 0.018 $h^{-1}$ in the presence of 10 $\mu$M PIPER) (*28*), (2) the low DNA concentrations (100 nM) used in the helicase unwinding experiments make reassembly unlikely, and (3) at low PIPER concentrations, there are few free PIPER molecules available for single-stranded DNA molecules to interact with, and because of their low affinity for PIPER, it is unlikely that single-stranded DNA molecules can compete with G-quadruplex DNA for PIPER. However, we cannot rule out the possibility that at high PIPER concentrations free PIPER molecules may facilitate reassembly of single-stranded DNA. Indeed, we observed that more G-quadruplex DNA was formed at higher PIPER concentrations than in the negative control in which neither PIPER or Sgs1 was added (Figure 2A; compare lanes 6−9 with lane 1), although this increase is more likely to come from the G-quadruplex stabilization effect of PIPER (see Results section).

The helicase assay in the presence of organic compounds described here provides a new method of screening for G-quadruplex DNA-interactive compounds. This assay is a supplement to the DNA polymerase assay we reported previously (*34*), which tests the interactivity of compounds with the intramolecular G-quadruplex. Sgs1 helicase is able to unwind tetrameric parallel G-quadruplex DNA and dimeric antiparallel G-quadruplex DNA and, therefore, can be used to screen for compounds that bind specifically to these structures. Indeed, we tested several other groups of G-quadruplex-interactive compounds such as the cationic porphyrins (TMPyP3 and TMPyP4) and found that they not only inhibit the unwinding of G-quadruplexes by Sgs1 but also show some degree of specificity to either parallel or antiparallel G-quadruplexes (Han et al., manuscript submitted). With the identification of new helicases capable of unwinding G-quadruplex structures formed from different genomic regions, this assay could be used to identify compounds that are specific to different G-quadruplex structures formed in defined genomic regions. Subsequently, these compounds could be used to selectively target cellular events.

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