

## Inhibition of the Bloom's and Werner's Syndrome Helicases by G-Quadruplex Interacting Ligands<sup>†</sup>

Ji-Liang Li,<sup>‡</sup> R. John Harrison,<sup>§</sup> Anthony P. Reszka,<sup>§</sup> Robert M. Brosh, Jr.,<sup>||</sup> Vilhelm A. Bohr,<sup>||</sup> Stephen Neidle,<sup>§</sup> and Ian D. Hickson<sup>\*,‡</sup>

*Imperial Cancer Research Fund Laboratories, University of Oxford, Weatherall Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, U.K., CRC Biomolecular Structure Unit, Chester Beatty Laboratories, The Institute of Cancer Research, 237 Fulham Road, London SW3 6JB, U.K., and Laboratory of Molecular Genetics, National Institute on Aging, National Institutes of Health, 5600 Nathan Shock Drive, Baltimore, Maryland 21224*

*Received May 23, 2001; Revised Manuscript Received September 5, 2001*

**ABSTRACT:** G-Quadruplex DNAs are folded, non-Watson–Crick structures that can form within guanine-rich DNA sequences such as telomeric repeats. Previous studies have identified a series of trisubstituted acridine derivatives that are potent and selective ligands for G-quadruplex DNA. These ligands have been shown previously to inhibit the activity of telomerase, the specialized reverse transcriptase that regulates telomere length. The RecQ family of DNA helicases, which includes the Bloom's (BLM) and Werner's (WRN) syndrome gene products, are apparently unique among cellular helicases in their ability to efficiently disrupt G-quadruplex DNA. This property may be relevant to telomere maintenance, since it is known that the sole budding yeast RecQ helicase, Sgs1p, is required for a telomerase-independent telomere lengthening pathway reminiscent of the "ALT" pathway in human cells. Here, we show that trisubstituted acridine ligands are potent inhibitors of the helicase activity of the BLM and WRN proteins on both G-quadruplex and B-form DNA substrates. Inhibition of helicase activity is associated with both a reduction in the level of binding of the helicase to G-quadruplex DNA and a reduction in the degree to which the G-quadruplex DNA can support DNA-dependent ATPase activity. We discuss these results in the context of the possible utility of trisubstituted acridines as antitumor agents for the disruption of both telomerase-dependent and telomerase-independent telomere maintenance.

The RecQ family of DNA helicases is highly conserved in evolution and has representatives in all organisms that have been analyzed to date (1, 2). In humans, there are five RecQ family members, defects in three of which give rise to disorders associated with genomic instability, namely, Bloom's syndrome (BS), Werner's syndrome (WS), and Rothmund-Thomson syndrome (RTS) (3–5). A variety of different chromosomal aberrations are present at an elevated frequency in cells isolated from individuals with these disorders (6–9). The hallmark of BS cells is hyper-recombination between both sister chromatids and homologous chromosomes, while WS cells characteristically show the occurrence of large chromosomal deletions (10, 11). Genomic instability has been noted in RTS cells, but precise details remain unclear (12). Cancer predisposition is also observed in all three of these disorders, although this is a particular feature of BS (6, 7). Significantly, BS individuals succumb to the full range of cancers seen in the normal population, but they do so several decades earlier in life than is generally expected. WS is primarily associated with the early appearance of several features of the normal aging

process, such as cataracts, osteoporosis, and graying and loss of hair (8). RTS is rather less well defined, but is characterized by skin and skeletal abnormalities (9). The BS and WS gene products, designated BLM and WRN, respectively, have been purified and characterized in vitro. As predicted from their sequences, BLM and WRN are DNA-dependent ATPases and ATP-dependent helicases that translocate along DNA in the 3'–5' direction (13–16). In addition, WRN possesses a 3'–5' exonuclease function, which is directed by a portion of the N-terminal domain of the protein that acts independently of the centrally located helicase domain (17–20).

Recent data indicate that BLM and WRN are atypical DNA helicases with respect to their DNA substrate specificity. Neither enzyme is capable of efficiently binding to or unwinding a blunt-ended DNA duplex, but both can unwind a similar substrate containing a 12-nucleotide, noncomplementary internal "bubble" or terminal "fork" (21). Moreover, both enzymes bind to a synthetic X-structure, which is a model for the Holliday junction recombination intermediate, even though this structure has four blunt-ended duplex arms (22, 23). Indeed, both BLM and WRN can efficiently promote branch migration of Holliday junctions and may, therefore, directly participate in certain genetic recombination reactions (22, 23). Previous studies have indicated, however, that perhaps the best substrate for BLM and WRN is a guanine-quadruplex, four-stranded DNA molecule (G4<sup>1</sup>

<sup>†</sup> Supported by the Imperial Cancer Research Fund and the Cancer Research Campaign, U.K.

<sup>\*</sup> To whom correspondence should be addressed. Telephone: +44-1865-222417. Fax: +44-1865-222431. E-mail: i.hickson@icrf.icnet.uk.

<sup>‡</sup> University of Oxford.

<sup>§</sup> The Institute of Cancer Research.

<sup>||</sup> National Institutes of Health.

DNA), with all four strands being held together by Hoogsteen hydrogen bonds (24, 25). Quadruplexes can form in vitro by interactions within or between DNA strands in molecules containing runs of guanine residues. They can exist in a variety of forms distinguished by strand orientation and stoichiometry (26).

Although there is no conclusive proof that G4 DNA can form in vivo, there are a number of loci within the human genome that contain guanine-rich sequences that readily form stable G4 DNA structures in vitro. The best characterized of these guanine-rich motifs are those that can form within telomeric repeat DNA, the ribosomal DNA gene cluster, immunoglobulin heavy chain gene switch regions, and the *c-myc* gene promoter (26). If it is assumed that G4 DNA structures form in vivo, even in a transient manner, they have the potential to influence normal chromosomal processes such as DNA replication, transcription, and recombination. The finding that some cellular proteins can interact selectively with G4 DNA implies that the formation of these structures may be exploited for normal DNA metabolism. Indeed, certain proteins, such as *Saccharomyces cerevisiae* Rap1p and Hop1p, not only bind G4 DNA structures but also promote their stable assembly (27).

RecQ helicases have connections with telomeric DNA structures in addition to their apparent preference for utilizing G4 DNA as a substrate for their helicase function. For example, WS is characterized at the cellular level not only by genomic instability but also by an abnormally short life span in culture (8). Primary fibroblasts from WS individuals undergo premature replicative senescence compared to normal controls, although this does not appear to be associated with any acceleration in the rate of telomere loss (28). Ectopic expression of telomerase can overcome the premature senescence in WS cells (28, 29), suggesting that in WS cells activation of the senescent state nevertheless involves signals originating from eroding telomeres. The budding yeast homologue of BLM and WRN, encoded by the *SGS1* gene (30, 31), also plays a role in telomere maintenance. Recent data indicate that yeast cells lacking telomerase maintain their telomeres via two genetically distinct pathways that depend on genetic recombination functions; both require *RAD52*, but *RAD50* and *RAD51* define separate pathways (32–34). The *RAD50*-dependent process, in which Sgs1p has been shown to act (32, 33), is mechanistically similar to the human “ALT” (for alternative lengthening of telomeres) pathway (35), in that telomeres maintained by this process are generally very long and contain variable amounts of TG<sub>1–3</sub> repeat sequence.

The propensity of guanine-rich telomeric repeat DNA to form quadruplex structures has attracted a great deal of attention because of the potential for this to be exploited as an antitumor therapy (36–38). The basis for this hypothesis is that in 80–85% of tumor cells telomere length is maintained, effectively indefinitely, by the enzyme telomerase. This specialized reverse transcriptase is generally not expressed in somatic cells. The absence of telomerase leads to progressive telomere erosion, and eventually to cellular senescence and chromosomal end-to-end fusions when telo-

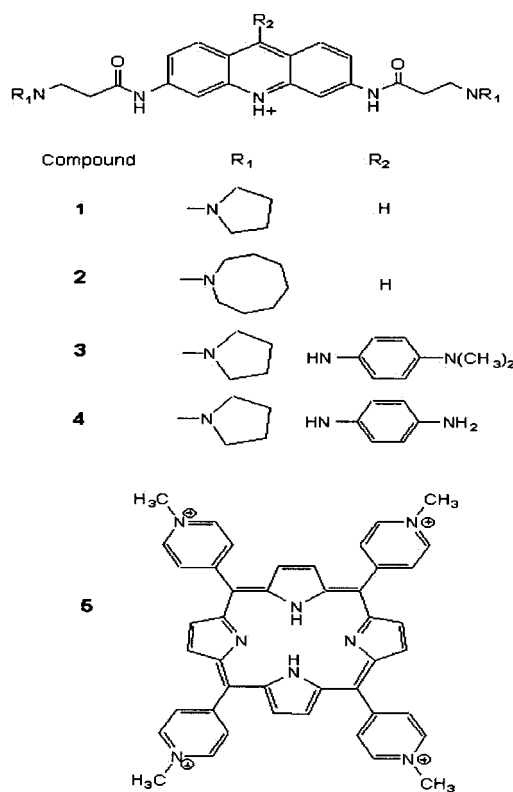


FIGURE 1: Structures of the substituted acridine and porphyrin compounds used in this study.

meres erode beyond a critical point (39). Telomerase contains an endogenous RNA component, which hybridizes with the single-stranded 3' end template stretch of telomeric DNA sequence, to which further guanine repeats are then added. Folding of this single-stranded telomeric region into G-quadruplex structures is known to halt telomerase-mediated synthesis (40), presumably because of interference with the essential DNA–RNA hybridization step during telomere elongation. It has been shown that several classes of G4 DNA interacting ligands can also inhibit telomerase, possibly by promoting the DNA folding process in a chaperone-type manner (41–51). These compounds are typified by the disubstituted acridine compound **1** (44, 45) and the tetra-*N*-methylpyridylporphyrin (TMPy) **5** (48, 49) (Figure 1), which are active against the enzyme in the low micromolar range. Most of these telomerase inhibitors also show general cellular cytotoxicity at an equivalent level. We have recently devised (52) a new series of 3,6,9-trisubstituted acridines that has significant (~40-fold) selectivity for binding to G4 DNA compared to regular B-form duplex DNA, and has approximately 100-fold greater potency against telomerase than has the disubstituted acridine **1**.

In this study, we have analyzed whether these di- and trisubstituted acridines have any inhibitory effect on the ability of the human BLM and WRN helicases to disrupt G4 DNA structures. A disubstituted acridine **2** that was previously found to be inactive against telomerase and to have lower quadruplex affinity (44–46, 52) was also examined. A previous study has shown that the quadruplex-binding ligand PIPER (50, 51) can inhibit the unwinding of G4 DNA by the Sgs1 helicase (53). We show here that the acridine-based compounds have potent in vitro inhibitory

<sup>1</sup> Abbreviations: BLM, Bloom's syndrome gene; WRN, Werner's syndrome gene; ALT, alternative lengthening of telomeres; G4, guanine quadruplex; DTT, dithiothreitol.

activity against the human BLM and WRN helicases. This inhibitory effect is not, however, dependent upon G4 DNA, since we also show that substituted acridines can inhibit BLM- and WRN-catalyzed unwinding of B-form DNA substrates.

## MATERIALS AND METHODS

**Proteins.** Recombinant human BLM protein was overexpressed in *S. cerevisiae* and purified by  $\text{Ni}^{2+}$  chelate affinity chromatography, as described previously (13, 54). His-tagged recombinant human WRN protein was expressed in Sf9 insect cells, and purified as described by Orren et al. (55). *Escherichia coli* UvrD protein was a kind gift of S. Matson (University of North Carolina, Chapel Hill, NC). T4 polynucleotide kinase was obtained from New England BioLabs. Protein amounts are indicated in moles of monomer.

**DNA-Binding Ligands.** The syntheses and characterizations of the di- and trisubstituted acridine derivative [compounds **1** ( $M_r = 568.97$ ), **2** ( $M_r = 653.14$ ), **3** ( $M_r = 703.14$ ), and **4** ( $M_r = 675.09$ ) (Figure 1)] have been described previously (44–46, 52). All of the compounds were employed as HCl salts and dissolved in distilled water. The tetra-*N*-methylpyridylporphyrin, compound **5** ( $M_r = 1363$ ), was purchased from Sigma as the tosylate salt.

**DNA Helicase Substrates.** G4-TP substrate was prepared as described by Sun et al. (24). The TP sequence is a consensus repeat from the murine immunoglobulin S $\gamma$ 2b switch region. The synthetic, four-way X-junction (HJX12) was made by annealing four 50-mer oligonucleotides (X12-1, X12-2, X12-3, and X12-4) as described previously (22). X12-1 was 5'- $^{32}\text{P}$ -labeled using T4 polynucleotide kinase before annealing with the other three oligonucleotides. Blunt-ended double-stranded DNA (DS52) was made from oligonucleotides BL3 and BL4 (22). Blunt-ended double-stranded DNA with a 12-nucleotide bubble at its center (bub12) was prepared by annealing oligonucleotides X0 (5'-GACGCTGCCGAATTCTGGCTTGCTCGGACATCTTTGCCCCACGTTGACCCG-3') and BU12 (5'-CGGGTCAACGTGGGCAAAGCCAATGCGATCGGCCAGAATTCGGCAGCGTC-3'). The forked DNA structure was made from two 50-mer oligonucleotides (X12-1 and X12-2), as described previously (22). All DNA substrates were purified following electrophoresis on 10% polyacrylamide gels.

**DNA Helicase Assays.** DNA helicase reaction mixtures (20  $\mu\text{L}$ ) contained 20 mM Tris-HCl (pH 7.5), 2 mM  $\text{MgCl}_2$ , 2 mM ATP, 100  $\mu\text{g}/\text{mL}$  BSA, 1 mM dithiothreitol (DTT), 1 nM DNA substrate, and the amounts of BLM, WRN, or UvrD protein and DNA-binding ligand indicated in the figure legends. Reactions were initiated by the addition of the appropriate helicase; the mixtures were incubated at 37 °C for various times, and the reactions were terminated by the addition of 2  $\mu\text{L}$  of 0.5 M EDTA. The products were resolved on 10% nondenaturing polyacrylamide gels run at 4 °C, and radioactive bands were visualized using a PhosphorImager (Molecular Dynamics) or by autoradiography. All helicase data are representative of at least three independent experiments.

**Electrophoretic Mobility Shift Assays.** Proteins and  $^{32}\text{P}$ -end-labeled DNA substrates were incubated at room temperature for 20 min in reaction buffer containing 20 mM triethanolamine-HCl (pH 7.5), 2 mM  $\text{MgCl}_2$ , 2 mM adenos-

ine 5'-[ $\gamma$ -thio]triphosphate (ATP $\gamma\text{S}$ ), 100  $\mu\text{g}/\text{mL}$  BSA, 1 mM DTT, 10 nM BLM/WRN protein, and the amounts of DNA-binding ligand indicated in the figure legends. Protein–DNA complexes were then fixed by incubation with 0.25% glutaraldehyde at 37 °C for 10 min, and the products were resolved on 5% nondenaturing polyacrylamide gels. Free DNA and DNA–protein complexes were observed by autoradiography.

**ATPase Assays.** ATPase activity was determined by the level of release of  $^{32}\text{P}_i$  from [ $\gamma$ - $^{32}\text{P}$ ]ATP. The reaction mixture (140  $\mu\text{L}$ ) contained 20 mM Tris-HCl (pH 7.5), 2 mM  $\text{MgCl}_2$ , 100  $\mu\text{g}/\text{mL}$  BSA, 1 mM DTT, DNA cofactor (either duplex oligonucleotide DS52 or TP-G4 DNA; either at 200 nM nucleotides), 0.2 mM ATP, 50 nCi of [ $\gamma$ - $^{32}\text{P}$ ]ATP (3000 Ci/mmol), 5 nM BLM/WRN protein, and 2.5  $\mu\text{M}$  DNA-binding ligand. Reactions were initiated by the addition of BLM/WRN protein, and reaction mixtures were incubated at 37 °C for up to 90 min. At the indicated time points, 20  $\mu\text{L}$  of the reaction mixture was collected and mixed with 10  $\mu\text{L}$  of 0.5 M EDTA to stop the reaction. One microliter of each reaction mixture was spotted onto CEL300PEI/UV $_{254}$  (Polygram) thin-layer chromatography plates, which were rinsed in 100% methanol before separation of  $^{32}\text{P}_i$  from [ $\gamma$ - $^{32}\text{P}$ ]ATP in buffer containing 0.8 M LiCl and 0.8 M acetic acid. The products were visualized using a PhosphorImager or by autoradiography, and the level of release of  $^{32}\text{P}_i$  was calculated by the formula % hydrolysis =  $100P/(P + S)$ , where  $P$  is the amount of product ( $^{32}\text{P}_i$ ) and  $S$  is the amount of substrate, after quantification using ImageQuant software (Molecular Dynamics). All ATPase data are representative of at least three independent experiments.

## RESULTS

To analyze whether the di- and trisubstituted acridine derivatives (Figure 1) could influence the unwinding of G4 DNA catalyzed by BLM or WRN, we generated and characterized, using an established method (24), a G4 DNA molecule representing the immunoglobulin heavy chain switch region (TP-G4). We have shown previously that the BLM and WRN helicases efficiently unwind TP-G4 and other G4 DNA structures in an ATP-dependent manner (21, 24).

Initial experiments showed that, at a fixed concentration of 2  $\mu\text{M}$ , compounds **3** and **4** were capable of near-complete inhibition of both BLM- and WRN-mediated unwinding of TP-G4 DNA. Accordingly, we analyzed in more detail the relative potency of compounds **1**–**4** as inhibitors of helicase-mediated disruption of TP-G4 DNA. Data obtained using a broad range of inhibitor concentrations (0–100  $\mu\text{M}$ ) showed that compounds **3** and **4** were significantly more potent than **1** and **2** at inhibiting BLM-catalyzed unwinding of TP-G4 DNA (data not shown). To extend this analysis and to determine  $\text{IC}_{50}$  values for each compound, additional unwinding reactions including BLM or WRN protein were performed using a narrower range of concentrations for each compound. These data indicate that compounds **3** and **4** were 15–30-fold more potent as BLM inhibitors than compounds **1** and **2** (Figure 2 and Table 1). The porphyrin molecule **5** also produced significant inhibition of BLM with an  $\text{IC}_{50}$  value that was slightly higher than that of **3** and **4** (Figure 2 and Table 1). Identical analyses were conducted substituting WRN for BLM, with similar results. Compounds **3**–**5** were



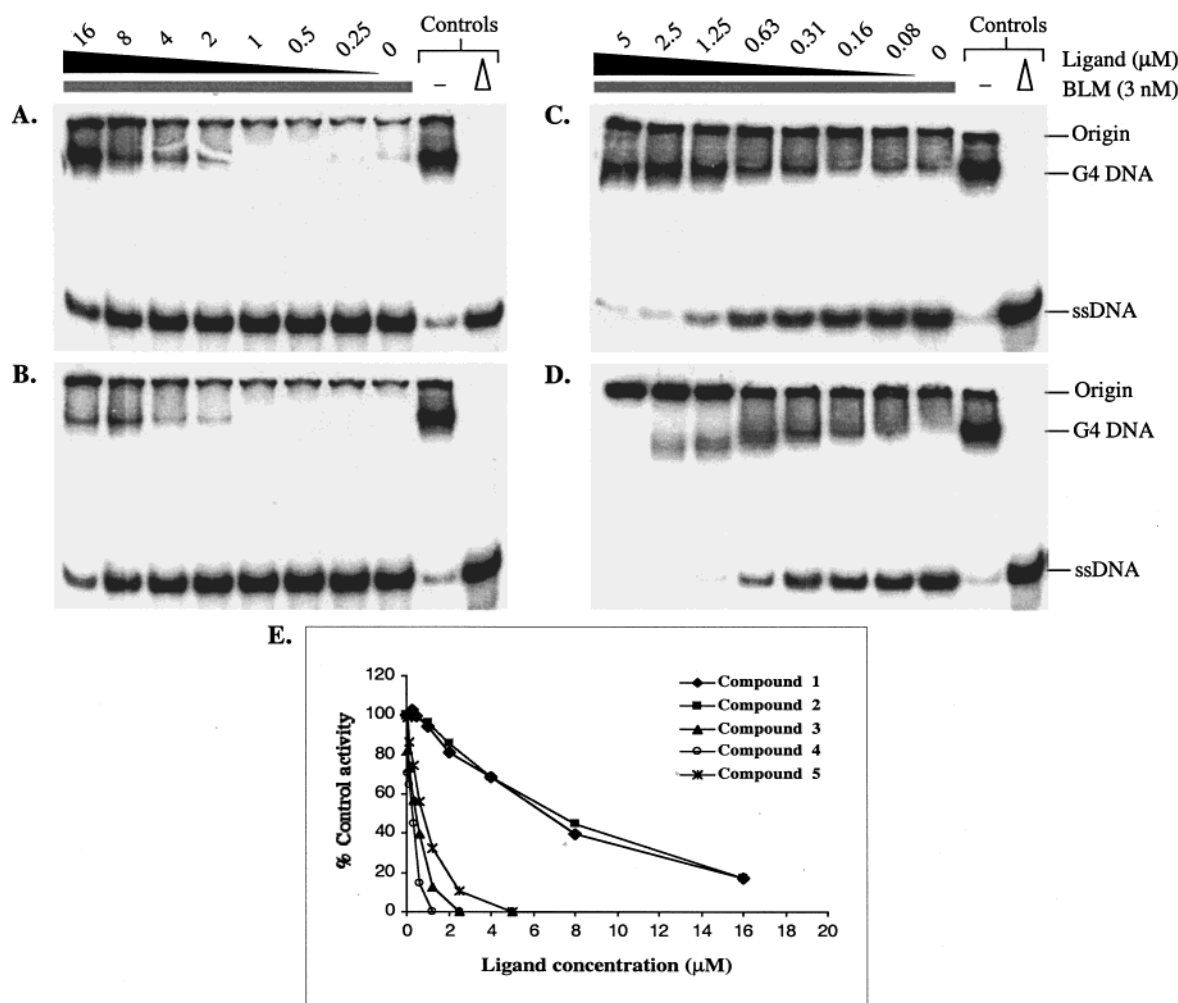


FIGURE 2: Determination of  $IC_{50}$  values for inhibition of BLM-catalyzed unwinding of TP-G4 DNA by G4 DNA-binding ligands. Panels A–D show autoradiograms of 10% polyacrylamide gels. All lanes, except the controls, contained 3 nM BLM, as indicated by the gray bar above the lanes. These lanes also included increasing concentrations of ligands (from 0 to 16  $\mu$ M) from right to left, as indicated above the lanes in black with the concentration of ligand in micromolar above. Note that the concentrations of ligand in panels A and B are higher than those in panels C and D. The control lanes contained no BLM and no ligand (–), to show the position of the G4 DNA, or no ligand ( $\Delta$ ) to show the position of the ssDNA product of the BLM action. The positions of the gel origin, G4 DNA substrate, and ssDNA product are indicated on the right in each case. Panels A–D contained a different ligand in each: (A) compound 1, (B) compound 2, (C) compound 3, and (D) compound 4. Panel E shows quantification of the data from panels A–D. Values represent the % control helicase activity (as represented by the 0  $\mu$ M ligand concentration lane). The primary data for compound 5 are not shown.

Table 1:  $IC_{50}$  Values for Inhibition of BLM- and WRN-Mediated Unwinding of the TP-G4 Substrate by G-Quadruplex Interacting Ligands

ligand	$IC_{50}$ ( $\mu$ M)		ligand	$IC_{50}$ ( $\mu$ M)	
	BLM	WRN		BLM	WRN
compound 1	6.51	1.38	compound 4	0.25	0.08
compound 2	7.20	1.10	compound 5	0.78	0.33
compound 3	0.40	0.11			

significantly more potent as WRN inhibitors than **1** and **2** (Figure 3). Moreover, WRN was consistently more sensitive to inhibition by these G4-interacting ligands than BLM (Table 1).

The data presented thus far indicate that the di- and trisubstituted acridine derivatives are capable of inhibiting the ability of BLM and WRN to unwind G4 DNA. To analyze the specificity of this inhibitory effect, we studied whether these acridines could also inhibit BLM- and/or WRN-catalyzed unwinding of two non-G4-containing DNA substrates. We have shown previously that both BLM and

WRN efficiently disrupt a synthetic X-junction, which mimics a Holliday junction recombination intermediate, and a blunt-ended duplex oligonucleotide substrate containing a centrally located, 12-nucleotide noncomplementary region (a so-called bubble substrate) (21–23). In this section of the study, compound **3** was used as a representative potent inhibitor of BLM and WRN. The data in Figure 4 indicate that compound **3** inhibited the ability of both BLM and WRN to unwind the synthetic X-junction and the bubble substrate. Moreover, this inhibitory effect was seen at ligand concentrations comparable to those required to inhibit unwinding of TP-G4 DNA by BLM and WRN (compare data in Figures 2 and 3 with those in Figure 4).

Next, we analyzed in more detail two aspects of the specificity of inhibition of BLM and WRN by substituted acridines. Because the analyses presented in Figures 2–4 compared G4 DNA and “alternate” DNA molecules such as the synthetic X-structure, we analyzed whether compound **3** could inhibit BLM- and WRN-mediated unwinding of a more conventional B-form DNA molecule. For this, we used

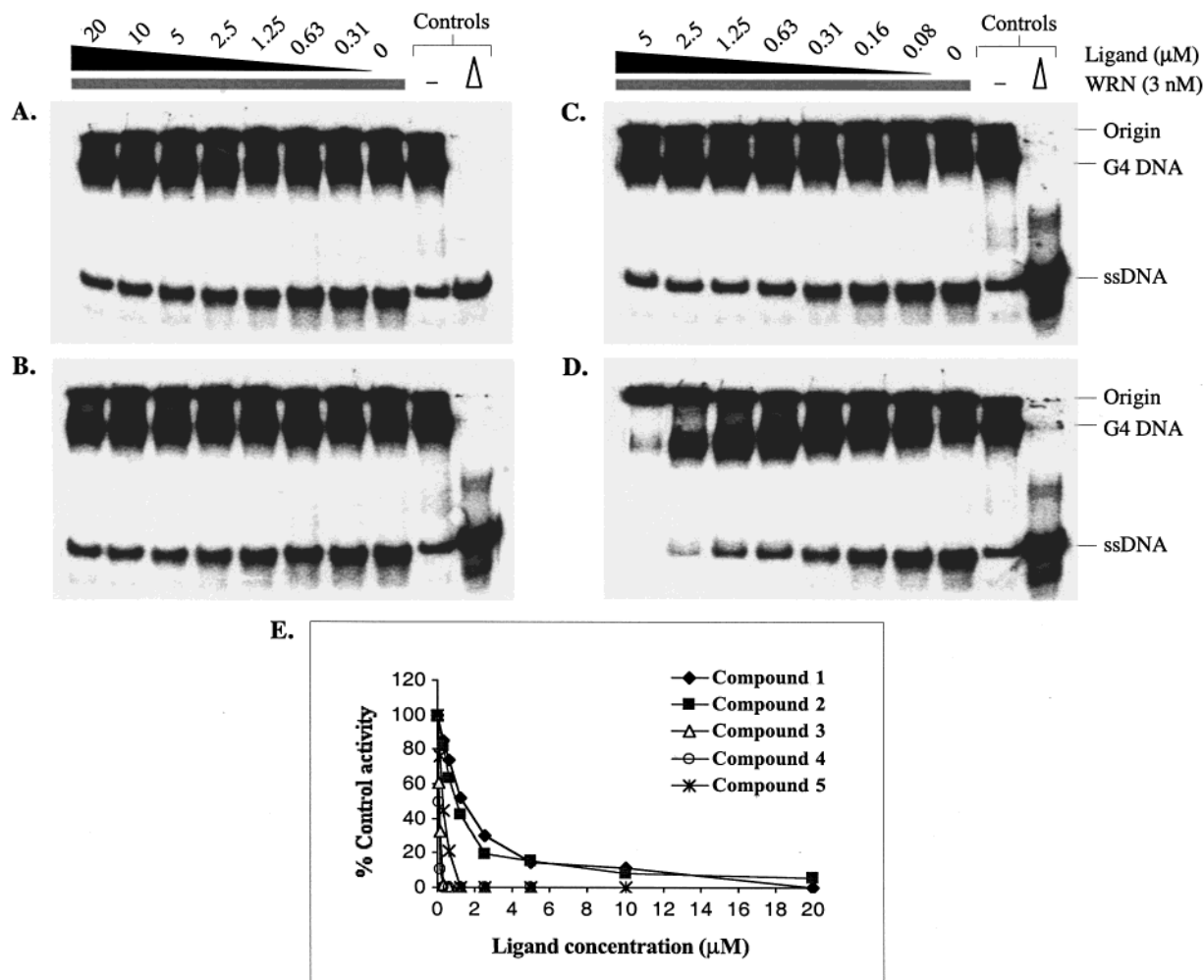


FIGURE 3: Determination of  $IC_{50}$  values for inhibition of WRN-catalyzed unwinding of TP-G4 DNA by G4 DNA-binding ligands. The processing of data and labeling of panels were as described in the legend of Figure 2. Note that the concentrations of ligand in panels A and B are higher than those in panels C and D. The primary data for compound 5 are not shown.

a forked DNA molecule, which is used extensively as a substrate for DNA helicases. The data in Figure 5 and Table 2 show that compound 3 potentially inhibited the unwinding of the forked DNA catalyzed by either BLM (Figure 5A) or WRN (Figure 5B). Next, we assessed whether the substituted acridines were acting as general DNA helicase inhibitors by studying the ability of compound 3 to block unwinding of the forked DNA catalyzed by a helicase that is not a member of the RecQ family. For this, we used *E. coli* UvrD protein. We found that while UvrD catalyzed robust unwinding of the forked substrate, compound 3, even at a concentration of 10  $\mu$ M, was unable to inhibit this unwinding activity. We conclude from this section of the study that substituted acridines inhibit the helicase activity of BLM and WRN, but not of the non-RecQ family helicase UvrD. Further, we conclude that these compounds are not specific in their inhibition of BLM and WRN in reactions that included G4 DNA, because they show a similar ability to inhibit the unwinding of conventional B-form DNA molecules.

Next, we analyzed two aspects of the mechanism by which acridines could be exerting an inhibitory effect on the helicase activities of BLM and WRN, namely, whether these ligands could inhibit the binding of the helicases to DNA and/or inhibit their DNA-dependent ATPase activities. First, we used electrophoretic mobility shift assays to assess whether

the inhibitory effect of the acridines was accompanied by a reduction in the extent to which BLM and/or WRN form a complex with the TP-G4 DNA substrate. For this section of the study, compound 3 was again used as a representative potent acridine compound, and was compared directly with porphyrin 5. In the absence of ligand, BLM and WRN caused retardation of the G4 substrate, producing two or three protein–DNA complexes that migrated a short distance into the gel (which were more pronounced for BLM than for WRN), as well as material that failed to migrate out of the wells (Figure 6). Addition of increasing concentrations of either compound 3 or porphyrin 5 had two effects. First, in the case of BLM, the intensity of the major BLM–G4 DNA complex declined in a ligand concentration-dependent manner. Second, and more significantly, the intensity of the band representing free G4 DNA increased correspondingly. For the reactions that included BLM and compound 3, there was an apparently reciprocal effect on the level of BLM–G4 DNA and free G4 DNA complexes. The results for compound 5 were less clear-cut, in that the amount of some BLM–G4 complexes declined, in line with the appearance of free G4 DNA, but the level of one protein–DNA complex actually increased at higher compound concentrations. In experiments that included WRN, the ligands had the effect both of reducing the level of WRN–G4 DNA complexes

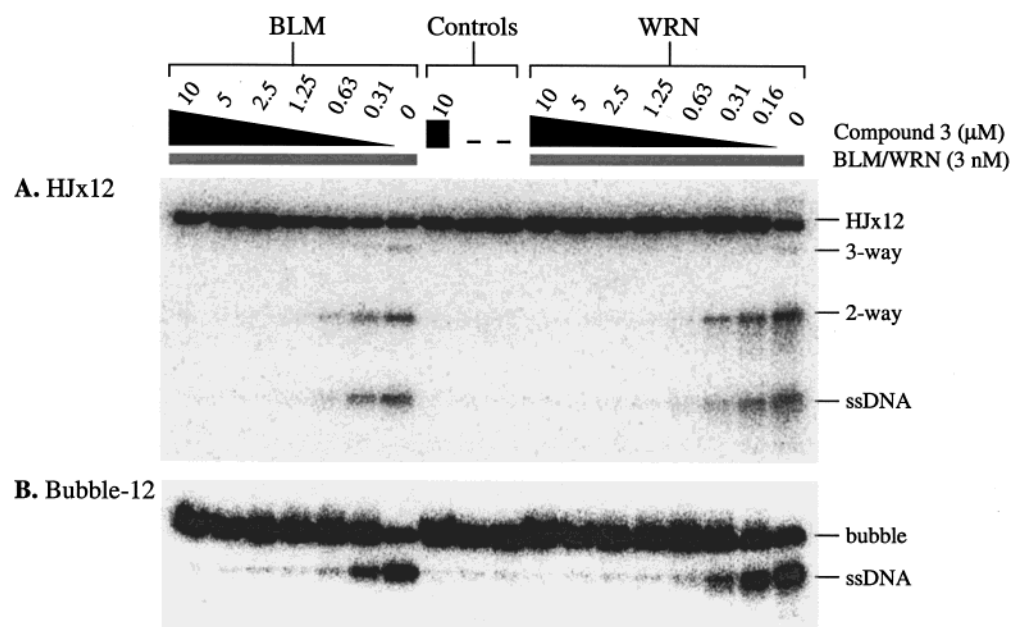


FIGURE 4: Effect of G4 DNA-binding ligands on the helicase activity of BLM and WRN using a synthetic X-junction substrate [HJx12 (A)] or a bubble substrate [Bubble-12 (B)]. Where indicated by the gray bar above the gels, reaction mixtures contained 3 nM BLM or WRN (as indicated above the labeling). Where indicated in black with concentrations (in micromolar) above the gels, reaction mixtures also contained ligand. The centrally located control lanes contained either no helicase and no ligand (–) or no helicase and 10  $\mu$ M ligand (black square numbered 10 above). In panel A (top gel), the positions of the four-way HJx12 substrate and the three products of unwinding (three-way junction, two-way junction, and ssDNA) are indicated on the right. In panel B (bottom gel), the positions of the bubble substrate and ssDNA product are indicated on the right.

and of causing disruption of the large complex that could not normally migrate from the gel wells (Figure 6), resulting in a corresponding increase in the level of free G4 DNA. Hence, we conclude that compounds **3** and **5** are capable of causing a reduction in the level of binding of BLM and WRN to G4 DNA.

We subsequently analyzed whether the acridine **3** could influence the level of DNA-dependent ATPase activity exhibited by BLM. Although BLM has no detectable activity as a helicase against fully duplex DNA (21), we have shown previously that double-stranded oligonucleotide cofactors are as efficient as single-stranded cofactors at stimulating the ATPase activity of BLM (13). Therefore, we compared the ability of duplex DNA and TP-G4 DNA to support ATPase activity in the presence and absence of compound **3**. The data in Figure 7 show that compound **3** had no effect on the ATPase activity of BLM in the presence of a double-stranded DNA cofactor, but caused a small, but significant, inhibition of ATPase activity in reactions that included TP-G4 DNA. These data are consistent with the known selectivity of compound **3** for binding G4 DNAs compared to B-form duplex DNA substrates (13).

## DISCUSSION

We have shown that di- and trisubstituted acridine derivatives are inhibitors of the unwinding activity Bloom's and Werner's syndrome gene products on a G4 DNA substrate. This is consistent with their known ability to bind to G4 DNA. Their ranking order of inhibitory potency is consistent with previous measurements of the level of binding to the human intramolecular quadruplex formed from a telomeric 22-mer DNA. The two trisubstituted compounds have markedly superior potency compared to the disubstituted ones

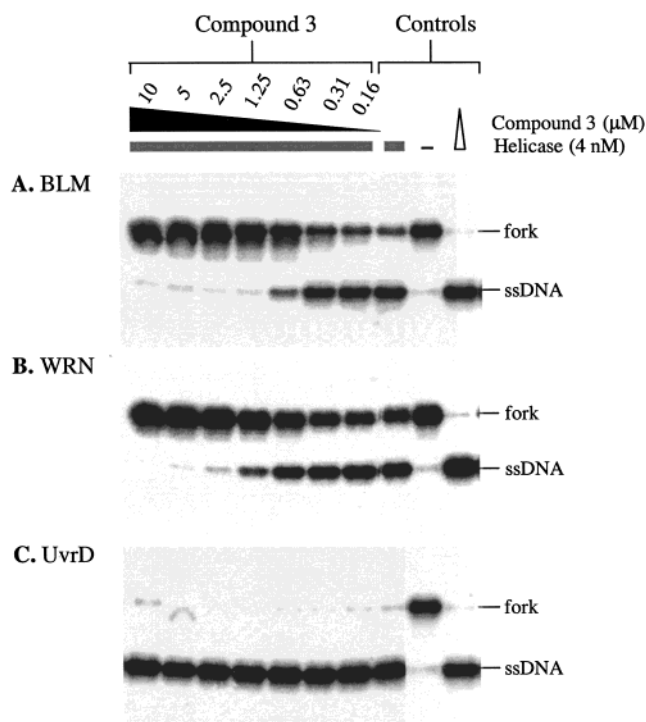


FIGURE 5: Effect of compound **3** on the unwinding of a forked DNA substrate by BLM (A), WRN (B), or UvrD (C). Where indicated by the gray bar above the gels, reaction mixtures contained 4 nM BLM, WRN, or UvrD. Where indicated in black with concentrations (in micromolar) above the gels, reaction mixtures also contained compound **3**. The control lanes contained helicase but no ligand (gray bar), no helicase and no ligand (–), or a sample of DNA heated to 95  $^{\circ}$ C for 3 min ( $\Delta$ ) to reveal the position of ssDNA. The positions of the forked DNA substrate and the ssDNA product are indicated on the right.

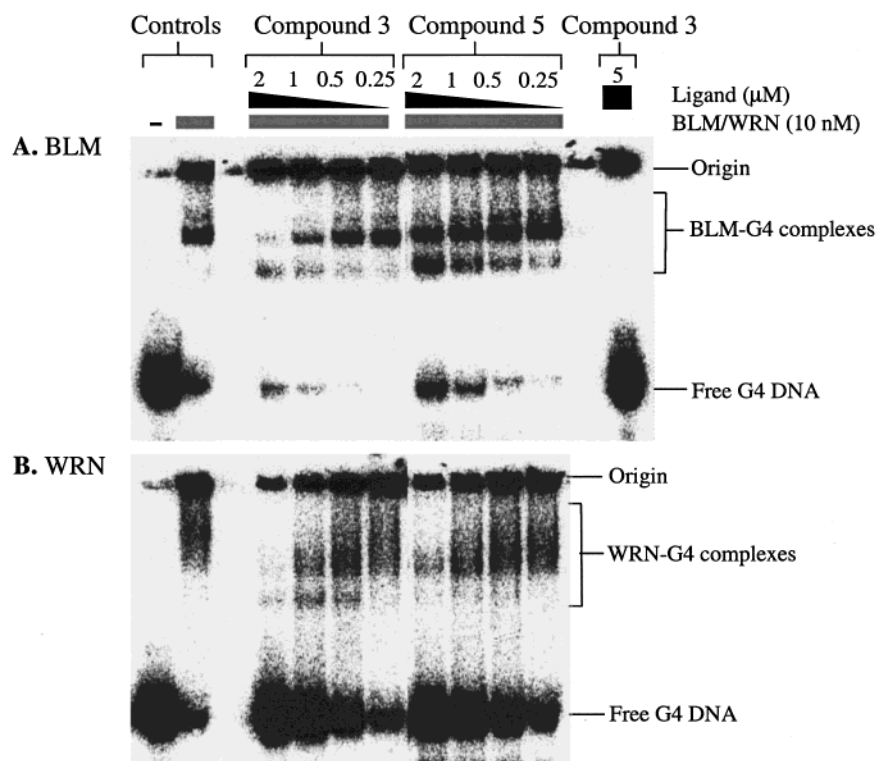


FIGURE 6: Electrophoretic mobility shift assay showing the effect of G4 DNA-binding ligands on the ability of BLM and WRN to form complexes with TP-G4 DNA. Panel A and panel B represent reaction mixtures containing BLM and WRN, respectively, as shown on the left. The control lanes contained DNA, but no helicase and no ligand (—), or 10 nM helicase and no ligand (gray bar above lane). These controls reveal the positions of the free G4 DNA and the protein–G4 complexes, respectively. The lane on the extreme right of panel A contained only DNA and 5  $\mu$ M compound 3, to indicate that the ligand did not generate a specific retarded product. All other lanes contained BLM or WRN at 10 nM, together with increasing concentrations (in micromolar) of G4 DNA-binding ligand (either compound 3 or compound 5, as indicated by the black bars above the lanes). The positions of the free G4 DNA, the protein–G4 DNA complexes, and the gel origin are shown to the right of each panel.

Table 2: IC<sub>50</sub> Values for Inhibition of BLM- and WRN-Mediated Unwinding of Various DNA Substrates by Compound 3

ligand	IC <sub>50</sub> ( $\mu$ M)		ligand	IC <sub>50</sub> ( $\mu$ M)	
	BLM	WRN		BLM	WRN
TP-G4	0.40	0.11	bubble	0.25	0.25
X-junction	0.55	0.40	fork	0.45	0.85

(44, 45, 52). Inhibition of helicase function by the acridines was accompanied by a reduction in the level of binding of BLM and/or WRN proteins to a G4 DNA structure, and by an inhibition of DNA-stimulated ATPase activity. Perhaps more surprisingly, the trisubstituted ligands also inhibited BLM- and WRN-catalyzed unwinding of three non-G4 DNA substrates. Despite the fact that two of these non-G4 substrates, the four-way Holliday-type junction and the bubble, are atypical in that they contain complex DNA structural features and are blunt-ended, compound 3 also inhibited BLM- and WRN-catalyzed unwinding of a standard B-form DNA helicase substrate, the forked partial duplex. This suggests that these compounds may have selectivity for DNA structures with a variety of nonduplex features, not just quadruplexes.

Sgs1p, the budding yeast homologue of BLM and WRN, plays a role in a recombination-dependent telomere maintenance pathway that is revealed in telomerase-deficient yeast cells (32–34). This *RAD50*- and *RAD52*-dependent pathway is at least superficially similar to the ALT pathway for telomere maintenance in immortalized human cells lacking telomerase, in that it mediates telomere length maintenance

through the addition of terminal TG<sub>1–3</sub> repeat units. The precise mechanism by which these TG repeats are added is not clear at this stage, although one possible mechanism is through the copying of TG units located on other chromosome ends (or extrachromosomally) following the priming of DNA synthesis from within a D-loop in a process resembling break-induced replication (see ref 56 for details). Although it is not yet clear whether any of the human RecQ helicases plays a role in telomere length maintenance, several lines of evidence suggest WRN as a possible candidate for such a role. First, primary WS fibroblasts undergo premature replicative senescence compared to normal control cells (28). Second, ectopic expression of telomerase in WS cells leads to immortalization (28, 29), indicating that telomerase erosion or telomere length maintenance defects underly this premature senescence. Third, expression of WRN can partially alleviate the telomere length maintenance defects of *sgs1* $\Delta$  mutants (34). Fourth, WRN interacts physically and functionally with the Ku70–Ku80 heterodimeric complex found at telomeres (57, 58). Nevertheless, it is possible that BLM or any of the other human RecQ family members plays a role at telomeres distinct from that of WRN. One circumstantial piece of evidence to suggest that this might be the case is that BLM has been shown to be a component of the specialized PML nuclear bodies that appear to localize to the sites of telomeric DNA in those cells in which telomeres are maintained by the ALT pathway (59).

We suggest that trisubstituted acridines have potential as novel antitumor agents. Most nondividing cells from somatic



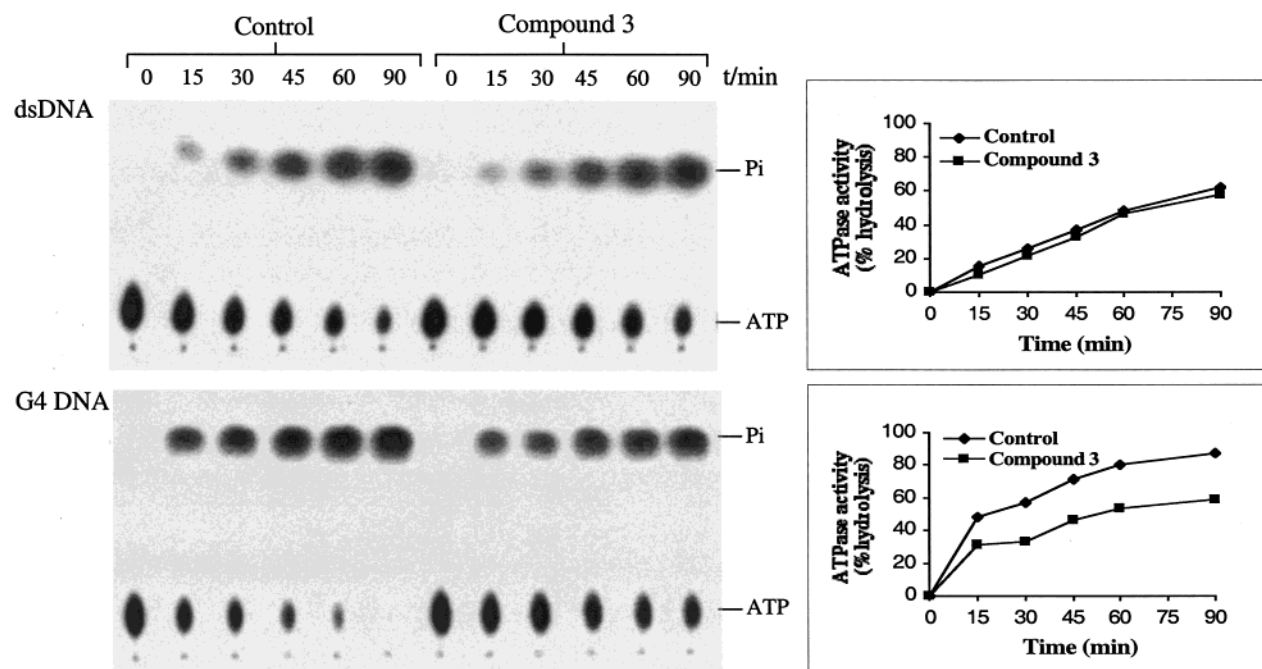


FIGURE 7: Effects of G4 DNA-binding ligand compound **3** on the ATPase activity of BLM protein. The panels on the left show phosphorimages of TLC plates representing time courses of BLM ATPase activity (5 nM enzyme). Incubation times are shown above the lanes. Reaction mixtures contained either no ligand (labeled control above) or 2.5  $\mu$ M compound **3** as indicated above. The positions of ATP and free  $P_i$  are shown to the right of each panel. In the top and bottom panels, dsDNA (200 nM) and TP-G4 DNA (200 nM), respectively, were used as cofactors, as indicated at the left. The graphs on the right show quantification of the data in each case.

tissues have no requirement for the enzyme telomerase, since telomeres generally only shorten as a result of a failure to adequately replicate to the very tip of linear chromosomes. However, proliferating cells, such as pluripotent stem cells or neoplastic cells, have a requirement to prevent such replication-dependent telomere loss. This requirement is generally fulfilled by telomerase, but approximately 10–20% of neoplastic cells maintain their telomeres through a recombination-mediated process termed ALT (35). In ALT cells, telomeres are of variable length and are often quite unstable, but nevertheless, such cells are immortal and can proliferate continuously in culture. Hence, an antitumor therapy designed to interfere with telomere maintenance may be ineffective in a minority of ALT tumors (such as certain osteosarcomas) if it is targeted solely at the telomerase enzyme itself. We have recently designed (52) ligands **3** and **4** to be selective binders of telomeric quadruplex DNA, and thus to be potent inhibitors of the telomerase enzyme complex. We now show here that they are also potent inhibitors of the unwinding activity of RecQ family helicases, when using both G4 DNA and regular B-form DNA as substrates. The X-junction is a model for the Holliday junction structure and was used because of the putative role of RecQ helicases in homologous recombination events. Thus, the inhibition by these ligands could lead to a decrease in the extent of recombination-directed telomere lengthening. These agents may have thus the potential to show a broader spectrum of antitumor activity than had hitherto been assumed, because of their potential to disrupt both telomerase-dependent and the telomerase-independent pathways for telomere maintenance. Hence, they may be able to inhibit proliferation of both telomerase-maintained and ALT-maintained tumors. This would give dual-function quadruplex and junction-targeted agents such as compounds **3** and **4** an important clinical advantage over pure active site or template-

directed telomerase inhibitors, especially if most tumors contain a small clonal population that have ALT characteristics. We have shown recently that compound **3** has in vivo activity against an ovarian tumor model, which is telomerase-positive (60). Cell-based and appropriate in vivo experiments are currently underway to examine whether compounds **3** and **4** and their analogues have activity in ALT cells and tumors.

## ACKNOWLEDGMENT

We thank members of the Bohr, Hickson, and Neidle groups, together with Dr. Lloyd Kelland, for useful discussions, Dr. Chris Norbury for critical reading of the manuscript, and Dr. S. Matson for the UvrD protein.

## REFERENCES

- Karow, J. K., Wu, L., and Hickson, I. D. (2000) *Curr. Opin. Genet. Dev.* 10, 32–38.
- Chakraverty, R. K., and Hickson, I. D. (1999) *BioEssays* 21, 286–294.
- Ellis, N. A., Groden, J., Ye, T. Z., Straughen, J., Lennon, D. J., Ciocchi, S., Proytcheva, M., and German, J. (1995) *Cell* 83, 655–666.
- Yu, C., Oshima, J., Fu, Y., Wijsman, E. M., Hisama, F., Alisch, R., Matthews, S., Najura, J., Miki, T., Ouais, S., Martin, G. M., Mulligan, J., and Schellenberg, G. D. (1996) *Science* 272, 258–262.
- Kitao, S., Shimamoto, A., Goto, M., Miller, R. W., Smithson, W. A., Lindor, N. M., and Furuichi, Y. (1999) *Nat. Genet.* 22, 82–84.
- German, J. (1993) *Medicine* 72, 393–406.
- German, J. (1995) *Dermatol. Clin.* 13, 7–18.
- Shen, J. C., and Loeb, L. A. (2000) *Trends Genet.* 16, 213–220.
- Vennos, E. M., and James, W. D. (1995) *Dermatol. Clin.* 13, 143–150.
- German, J., Crippa, L. P., and Bloom, D. (1974) *Chromosoma* 48, 361–366.



11. Fukuchi, K., Martin, G. M., and Monnat, R. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86, 5893–5897.
12. Lindor, N. M., Devries, E. M., Michels, V. V., Schad, C. R., Jalal, S. M., Donovan, K. M., Smithson, W. A., Kvols, L. K., Thibodeau, S. N., and Dewald, G. W. (1996) *Clin. Genet.* 49, 124–129.
13. Karow, J. K., Chakraverty, R. K., and Hickson, I. D. (1997) *J. Biol. Chem.* 272, 30611–30614.
14. Gray, M. D., Shen, J. C., Kamath-Loeb, A. S., Blank, A., Sopher, B. L., Martin, G. M., Oshima, J., and Loeb, L. A. (1997) *Nat. Genet.* 17, 100–103.
15. Suzuki, N., Shimamoto, A., Imamura, O., Kuromitsu, J., Kitao, S., Goto, M., and Furuichi, Y. (1997) *Nucleic Acids Res.* 25, 2973–2978.
16. Shen, J. C., Gray, M. D., Oshima, J., and Loeb, L. A. (1998) *Nucleic Acids Res.* 26, 2879–2885.
17. Huang, S., Li, B., Gray, M. D., Oshima, J., Mian, I. S., and Campisi, J. (1998) *Nat. Genet.* 20, 114–116.
18. Shen, J. C., Gray, M. D., Oshima, J., Kamath-Loeb, A. S., Fry, M., and Loeb, L. A. (1998) *J. Biol. Chem.* 273, 34139–34144.
19. Kamath-Loeb, A. S., Shen, J. C., Loeb, L. A., and Fry, M. (1998) *J. Biol. Chem.* 273, 34145–34150.
20. Suzuki, N., Shiratori, M., Goto, M., and Furuichi, Y. (1999) *Nucleic Acids Res.* 27, 2361–2368.
21. Mohaghegh, P., Karow, J. K., Brosh, R. M. J., Bohr, V. A., and Hickson, I. D. (2001) *Nucleic Acids Res.* 29, 2843–2849.
22. Karow, J. K., Constantinou, A., Li, J. L., West, S. C., and Hickson, I. D. (2000) *Proc. Natl. Acad. Sci. U.S.A.* 97, 6504–6508.
23. Constantinou, A., Tarsounas, M., Karow, J. K., Brosh, R. M., Bohr, V. A., Hickson, I. D., and West, S. C. (2000) *EMBO Rep.* 1, 80–84.
24. Sun, H., Karow, J. K., Hickson, I. D., and Maizels, N. (1998) *J. Biol. Chem.* 273, 27587–27592.
25. Fry, M., and Loeb, L. A. (1999) *J. Biol. Chem.* 274, 12797–12802.
26. Gilbert, D. E., and Feigon, J. (1999) *Curr. Opin. Struct. Biol.* 9, 305–314.
27. Muniyappa, K., Anuradha, S., and Byers, B. (2000) *Mol. Cell. Biol.* 20, 1361–1369.
28. Choi, D., Whittier, P. S., Oshima, J., and Funk, W. D. (2001) *FASEB J.* 15, 1014–1020.
29. Wyllie, F. S., Jones, C. J., Skinner, J. W., Haughton, M. F., Wallis, C., Wynford-Thomas, D., Faragher, R. G., and Kipling, D. (2000) *Nat. Genet.* 24, 16–17.
30. Gangloff, S., McDonald, J. P., Bendixen, C., Arthur, L., and Rothstein, R. (1994) *Mol. Cell. Biol.* 14, 8391–8398.
31. Watt, P. M., Louis, E. J., Borts, R. H., and Hickson, I. D. (1995) *Cell* 81, 253–260.
32. Huang, P., Pryde, F. E., Lester, D., Maddison, R. L., Borts, R. H., Hickson, I. D., and Louis, E. J. (2001) *Curr. Biol.* 11, 125–129.
33. Johnson, F. B., Marciniak, R. A., McVey, M., Stewart, S. A., Hahn, W. C., and Guarente, L. (2001) *EMBO J.* 20, 905–913.
34. Cohen, H., and Sinclair, D. A. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 3174–3179.
35. Dunham, M. A., Neumann, A. A., Fasching, C. L., and Reddel, R. R. (2000) *Nat. Genet.* 26, 447–450.
36. Mergny, J. L., and Helene, C. (1998) *Nat. Med.* 4, 1366–1367.
37. Han, H., and Hurley, L. H. (2000) *Trends Pharmacol. Sci.* 21, 136–142.
38. Neidle, S., and Read, M. A. (2001) *Biopolymers* (in press).
39. Holt, S. E., and Shay, J. W. (1999) *J. Cell Physiol.* 180, 10–18.
40. Zahler, A. M., Williamson, J. R., Cech, T. R., and Prescott, D. M. (1991) *Nature* 350, 718–720.
41. Sun, D., Thompson, B., Cathers, B. E., Salazar, M., Kerwin, S. M., Trent, J. O., Jenkins, T. C., Neidle, S., and Hurley, L. H. (1997) *J. Med. Chem.* 40, 2113–2116.
42. Perry, P. J., Gowan, S. M., Reszka, A. P., Polucci, P., Jenkins, T. C., Kelland, L. R., and Neidle, S. (1998) *J. Med. Chem.* 41, 3253–3260.
43. Perry, P. J., Reszka, A. P., Wood, A. A., Read, M. A., Gowan, S. M., Dosanjh, H. S., Trent, J. O., Jenkins, T. C., Kelland, L. R., and Neidle, S. (1998) *J. Med. Chem.* 41, 4873–4884.
44. Read, M. A., Wood, A. A., Harrison, J. R., Gowan, S. M., Kelland, L. R., Dosanjh, H. S., and Neidle, S. (1999) *J. Med. Chem.* 42, 4538–4546.
45. Harrison, R. J., Gowan, S. M., Kelland, L. R., and Neidle, S. (1999) *Bioorg. Med. Chem. Lett.* 9, 2463–2468.
46. Neidle, S., Harrison, R. J., Reszka, A. P., and Read, M. A. (2000) *Pharmacol. Ther.* 85, 133–139.
47. Mergny, J. L., Lacroix, L., Teulade-Fichou, M. P., Hounsou, C., Guittat, L., Hoarau, M., Arimondo, P. B., Vigneron, J. P., Lehn, J. M., Riou, J. F., Garestier, T., and Helene, C. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 3062–3067.
48. Izbicka, E., Wheelhouse, R. T., Raymond, E., Davidson, K. K., Lawrence, R. A., Sun, D., Windle, B. E., Hurley, L. H., and Von Hoff, D. D. (1999) *Cancer Res.* 59, 639–644.
49. Hurley, L. H., Wheelhouse, R. T., Sun, D., Kerwin, S. M., Salazar, M., Fedoroff, O. Y., Han, F. X., Han, H., Izbicka, E., and Von Hoff, D. D. (2000) *Pharmacol. Ther.* 85, 141–158.
50. Fedoroff, O. Y., Salazar, M., Han, H., Chemeris, V. V., Kerwin, S. M., and Hurley, L. H. (1998) *Biochemistry* 37, 12367–12374.
51. Han, H., Cliff, C. L., and Hurley, L. H. (1999) *Biochemistry* 38, 6981–6986.
52. Read, M., Harrison, R. J., Romagnoli, B., Tanious, F. A., Gowan, S. H., Reszka, A. P., Wilson, W. D., Kelland, L. R., and Neidle, S. (2001) *Proc. Natl. Acad. Sci. U.S.A.* 98, 4844–4849.
53. Han, H., Bennett, R. J., and Hurley, L. H. (2000) *Biochemistry* 39, 9311–9316.
54. Karow, J. K., Newman, R. H., Freemont, P. S., and Hickson, I. D. (1999) *Curr. Biol.* 9, 597–600.
55. Orren, D. K., Brosh, R. M., Jr., Nehlin, J. O., Machwe, A., Gray, M. D., and Bohr, V. A. (1999) *Nucleic Acids Res.* 27, 3557–3566.
56. Pagues, F., and Haber, J. E. (1999) *Microbiol. Mol. Biol. Rev.* 63, 349–404.
57. Li, B., and Comai, L. (2000) *J. Biol. Chem.* 275, 39800.
58. Cooper, M. P., Machwe, A., Orren, D. K., Brosh, R. M., Ramsden, D., and Bohr, V. A. (2000) *Genes Dev.* 14, 907–912.
59. Yeager, T. R., Neumann, A. A., Englezou, A., Huschtscha, L. I., Noble, J. R., and Reddel, R. R. (1999) *Cancer Res.* 59, 4175–4179.
60. Kelland, L. R., et al. (2001) (manuscript in preparation).

BI011067H