Supporting Information

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SI Materials and Methods

Proteins. Recombinant Werner syndrome (WRN) (1), RECQ1 (2), and Fanconi anemia group J (FANCJ) (3) were overexpressed using baculovirus expression systems and purified as previously described. Recombinant UvrD and RecQ were purchased from BioHelix and Abcam, respectively. Recombinant Bloom syndrome protein (BLM) and DnaB were kindly provided by Ian Hickson (University of Oxford, Oxford) and Daniel Kaplan (Vanderbilt University, Nashville, TN), respectively.

DNA Substrates. The 19-bp forked duplex DNA helicase substrate (4) and 3′ recessed duplex DNA exonuclease substrate (5) were prepared as previously described. Covalently closed M13 ssDNA was purchased from New England Biolabs.

Thiazole Orange Displacement Assays. In initial studies, Thiazole Orange titrations with the forked duplex substrate were performed to determine the saturating concentration of Thiazole Orange for subsequent experiments with NSC 19630 or Hoescht 33528. For dye displacement assays, Thiazole Orange (400 nM) was preincubated with 50 nM forked duplex DNA substrate in WRN helicase reaction buffer (*Materials and Methods*) with 2 mM ATP in a 50-μL reaction volume for 10 min at room temperature. Following this incubation, either NSC 19630 or Hoescht 33258 was added at the indicated concentrations and incubated for 15 min at 37 °C. Fluorescence (excitation 485 nm, emission 520 nm) was measured at the end of the 15-min incubation using a FluoSTAR OPTIMA (BMG Labtek).

ATPase Assays. Reaction mixtures (30 μL) contained 1 mM [^{32}P]γ-ATP (3,000 C_i/mmol) (Perkin-Elmer) in 30 mM Hepes (pH 7.4), 5% glycerol, 40 mM KCl, 100 μg/mL BSA, 8 mM $MgCl_2$, and the indicated concentrations of NSC 19630. Reactions were initiated by the addition of WRN (56 nM) and incubated at 37 °C for 0, 5, 10, 20, and 30 min. Aliquots (5 μL) were removed at the specified time points and quenched with the addition of 5 μL ATPase Stop Solution (6.7 mM ADP, 6.7 mM ATP, 33.3 mM EDTA). Two microliters of the quenched reaction mixture were spotted on a 10 × 20 cm sheet of PEI Cellulose (JT Baker) 1 cm from the bottom edge with a 1.5-cm space between each spot. The cellulose sheet was placed vertically in a chamber containing TLC running buffer (1 M formic acid, 0.8 M LiCl) until the buffer front reached the top of the sheet. The sheet was removed, wrapped in plastic, and exposed for 30 min on a phosphorimager screen. The screen was scanned in a Typhoon scanner (GE), and spots were quantitated. A graph of product (ATP hydrolyzed) per unit time was generated, and the slope of the fitted line was divided by the amount of WRN (nmol) to generate k_{cat} values for WRN ATP hydrolysis.

Exonuclease Assays. WRN was incubated with the indicated concentrations of NSC 19630 in a 10-μL reaction containing Exonuclease buffer [40 mM Tris (pH 8.0), 2 mM $MgCl_2$, 5 mM DTT, 100 μg/mL BSA, 1 mM ATP, and 0.25 nM 5′ tail DNA substrate]. Reactions were initiated by the addition of WRN (15 nM) and incubated at 37 °C for 20 min. Reactions were terminated by the addition of 10 μL formamide loading buffer followed by 3-min incubation at 95 °C. Reaction products were resolved on denaturing 14% polyacrylamide gels and visualized using a PhosphorImager.

WRN Silencing in HeLa Cells. The sequence of WRN siRNA was 5′-GTGTATAGTTACGATGCTAGTGATT-3′, and that of the control siRNA was 5′-UUCUCCGACGUGUCACGUUU-3′. siRNA was transfected using Lipofectamine 2000 following the manufacturer's protocol (Invitrogen). Cells were plated to 50–60% confluence in 10-cm dishes 24 h before transfection. siRNA (0.6 nmol) was mixed with 30 μL of Lipofectamine 2000 in 3 mL of Opti-MEM (Invitrogen). The mixture was added to cells that subsequently were incubated for 6 h. After 24 h, a second transfection was performed similarly. Seventy-two hours after the initial transfection, cells were harvested for preparing lysate or were treated with small-molecule compounds or DMSO at the indicated concentrations, and cell proliferation was measured using WST-1 reagent (Roche) as described in *Materials and Methods* for HeLa cells.

For experiments with siRNA-transfected cells that were allowed to recover WRN expression, WRN-depleted HeLa cells were grown for 2 wk after 48 h of initial siRNA-WRN transfection. Cells were harvested for preparing lysate or were treated with small-molecule compounds or DMSO at the indicated concentrations, and cell proliferation was measured using WST-1 reagent.

For lysate preparation, cells were washed twice with 1× PBS. RIPA buffer [10 mM sodium phosphate (pH 7.2), 300 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholate, 2 mM EDTA] was added to the cells, and the cells were incubated at 4 °C for 30 min. Cells were scraped, and the suspension was incubated further on ice for 30 min. Then the cell suspension was centrifuged at 18,500 × *g* for 10 min at 4 °C, and supernatant was collected. Twenty micrograms of the lysate were loaded on 8–16% SDS/PAGE. Protein was transferred onto a PVDF membrane, and the blot was probed with anti-WRN antibody (1:1,000) (Spring Valley Laboratories) as the primary antibody. For the secondary antibody, peroxidase-labeled anti-mouse IgG (1:1,000) (Vector) was used. The blot was developed using the ECL Plus Western Blot Detection Kit according to the manufacturer's protocol (Amersham). As a loading control, the blot was stripped and then reprobed with anti-actin antibody (1:5,000) (Sigma).

For analysis of the WRN expression levels in National Cancer Institute (NCI) 60 cancer lines, cell lysates for all the cell lines were prepared and analyzed for WRN protein by Western blot as described above. The percentage of WRN expressed in each cell line then was calculated relative to the amount of WRN expressed in HeLa as 100%. WRN protein levels in the NCI60 cell lines were analyzed relative to the pattern of NSC 19630 sensitivity using the COMPARE algorithm (6) via an interactive tool available at the NCI website (dtp.nci.nih.gov). The pattern of WRN protein expression was not significantly correlated with the pattern of cellular sensitivity to NSC 19630 (correlation coefficient = 0.17).

Cell Cycle Analysis by Flow Cytometry. HeLa cells were treated with 2 μM NSC 19630 or 1% DMSO as a control for 72 h. Cells then were prepared for flow cytometric analysis following the nuclear isolation media technique. Briefly, cells were washed with PBS free of Ca^{2+} and Mg^{2+}, trypsinized for 10–12 min, and harvested by centrifugation at 400 × *g* for 7 min at 4 °C. The cell pellet was resuspended in 0.5 mL FBS. Then 0.5 mL of detergent buffer, pH 7.4, (137 mM NaCl, 5.4 mM KCl, 0.5 mM KH_2PO_4, 0.3 mM Na_2HPO_4 $7H_2O$, 1.3 mM $CaCl_2$, 0.5 mM $MgCl_2$ $6H_2O$, 0.4 mM $MgSO_4$ $7H_2O$, 23.5 mM Hepes, 30 mM BSA, and 0.4% Nonidet P-40) was added, followed by 15 μL of 0.5 M RNase (Sigma)

solution. The sample was mixed by inversion and incubated at room temperature in the dark for at least 1 h before analysis. Samples then were analyzed on a FACScan (Becton Dickinson).

To determine the effect of WRN knockdown on the length of S phase, control siRNA or WRN siRNA-depleted HeLa cells were treated with 0.5 μM mimosine for 16 h. Cells then were washed with PBS and incubated with fresh medium for 8 h. At this time, mimosine-treated cells had reached mid to late S phase. Aliquots of cells were collected at the indicated time intervals and prepared for FACScan analysis as described above.

Detection of Ataxia Telangiectasia Mutated Phosphorylation upon NSC 19630 Cellular Exposure. HeLa cells were treated with either 1% DMSO or 2 μM NSC 19630 for the indicated time periods. Cells then were harvested by centrifugation at 1600 × *g* for 10 min at 4 °C, washed once with PBS, and resuspended in RIPA buffer [10 mM sodium phosphate (pH 7.2), 300 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 1% deoxycholate, and 2 mM EDTA] containing protease and phosphatase inhibitor mixture and were incubated at 4 °C for 30 min. Cells were incubated further on ice for 30 min and centrifuged at 18,500 × *g* for 10 min at 4 °C. The supernatant was collected, and 200 μg of the lysate was loaded on 4% SDS/PAGE. Following electrophoresis, protein was transferred onto a PVDF membrane, and blot was probed with anti-pATM Ser1981 antibody (1:500) (Santa Cruz). For the secondary antibody, peroxidase-labeled anti-mouse IgG (1:1,000) (Vector) was used. The blot was developed using the ECL Plus Western Blot Detection Kit following the manufacturer's protocol (Amersham). As a control, the blot was stripped and then reprobed with anti-ataxia telangiectasia mutated (ATM) antibody (1:500) (Millipore).

Colony Survival Assays. To measure colony survival, 250 HeLa cells per well were seeded in a six-well plate 24 h before treatment. Cells then were exposed to the indicated concentrations of NSC 19630 or 1% DMSO as a control for 72 h at 37 °C. Following exposure, cells were washed twice with 1× PBS and incubated in a regular maintenance medium (DMEM, 10% FBS) at 37 °C for 7 d. Colonies formed were fixed in methanol, stained with methylene blue (Sigma), and counted to determine percentage survival relative to the DMSO control.

DNA Synthesis by EdU Incorporation. HeLa cells (25,000 cells per well) were treated with 2 μM NSC 19630 or with DMSO as a control for 72 h in four-well chamber slides (Lab-Tek). EdU (10 μM) (Invitrogen) was added to the culture medium, and slides were incubated at 37 °C for 6 h. Cells were washed twice with 1× PBS and fixed with 3.7% formaldehyde at room temperature for 15 min. Following fixation, cells were washed twice with 3% BSA and treated with 0.5% Triton X-100 at room temperature for 20 min. After permeabilization, cells were washed twice with 3% BSA. Then 0.5 mL Click-iT reaction mixture (1× Click-iT reaction buffer, CuSO4, Alexa Fluor 594 azide, and reaction buffer additive) (Invitrogen) was added to each well and was incubated at room temperature in the dark for 30 min. Cells were washed once with 3% BSA and coated with Prolong Gold Anti-Fade reagent containing DAPI (Invitrogen). Coverslips were placed on chamber slides, and cells were cured at room temperature in the dark for 24 h. Immunofluorescence analyses were performed with a Zeiss LSM 510 META inverted Axiovert 200 M laser scan microscope with a Plan-Apochromat 100× 1.4-numerical-aperature oil immersion differential interference contrast objective lens. Images were captured with a CCD camera and analyzed using the LSM Browser software package.

γ-H2AX Foci Detection by Immunofluorescence. HeLa cells were treated with 2 μM NSC 19630 or 1% DMSO as a control for 72 h in slide chambers with four wells (Lab-Tek). Cells then were washed twice with 1× PBS and fixed with formaldehyde (3.7%) at room temperature for 15 min. Fixed cells were washed four times with 1× PBS and treated with 0.5% Triton X-100 (Sigma) at room temperature for 5 min. Cells were washed four times with 1× PBS containing 0.5% Tween-20 and blocked with 10% goat serum (Sigma) overnight at 4 °C. Cells were washed four times with 0.1% Tween-20 and incubated with mouse anti–γ-H2AX monoclonal antibody (1:300) (Upstate) or mouse proliferating cell nuclear antigen (PCNA) F-2 monoclonal antibody (1:500) (Santa Cruz) overnight at 4 °C. After four washes with 0.1% Tween-20, cells were incubated with Alexa Fluor 488-conjugated donkey anti-mouse IgG (1:400) (Invitrogen) at room temperature for 2 h. Cells were washed four times with 0.1% Tween-20 and coated with Prolong Gold Anti-Fade reagent (Invitrogen) containing DAPI. Coverslips were placed on the chamber slides, and cells were cured at room temperature in the dark for 24 h. Immunofluorescence analyses were performed as described above.

To determine the effect on γ-H2AX foci formation of the combined exposure of HeLa cells to TPT and NSC 19630, cells were cotreated with the indicated compound concentrations at 37 °C for 72 h. Cells were fixed with formaldehyde and processed for immunostaining to detect γ-H2AX foci as described above.

Cellular Apoptosis Assays. HeLa cells were exposed to the indicated concentrations of NSC 19630 or 1% DMSO as a control for 72 h at 37 °C. To assay apoptosis, the cytoplasmic histone-associated DNA fragments indicative of ongoing apoptosis were measured quantitatively using the cell death detection ELISA PLUS photometric enzyme assay (Roche).

1. Sharma S, et al. (2004) WRN helicase and FEN-1 form a complex upon replication arrest and together process branch migrating DNA structures associated with the replication fork. *Mol Biol Cell* 15:734–750.
2. Sharma S, et al. (2005) Biochemical analysis of the DNA unwinding and strand annealing activities catalyzed by human RECQ1. *J Biol Chem* 280:28072–28084.
3. Gupta R, et al. (2005) Analysis of the DNA substrate specificity of the human BACH1 helicase associated with breast cancer. *J Biol Chem* 280:25450–25460.
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6. Paull KD, et al. (1989) Display and analysis of patterns of differential activity of drugs against human tumor cell lines: Development of mean graph and COMPARE algorithm. *J Natl Cancer Inst* 81:1088–1092.

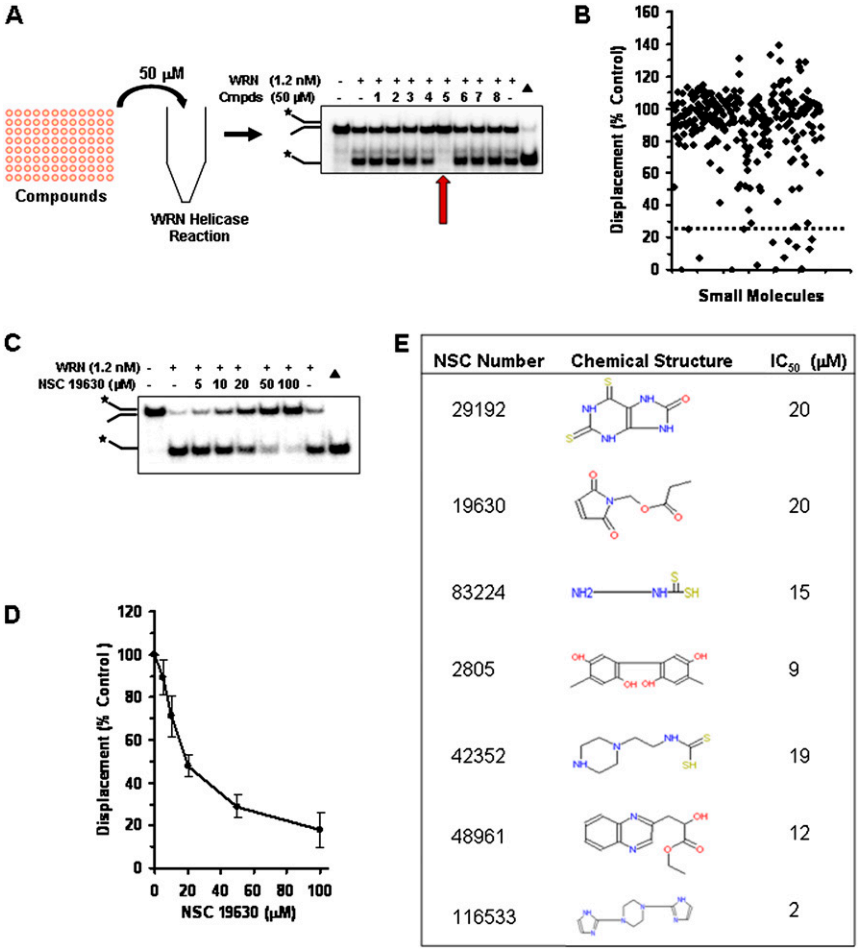

Fig. S1. Assay for screening small-molecule modulators of WRN helicase activity. (*A*) Schematic of in vitro radiometric assay used to screen NCI Diversity Set compounds for modulation of WRN helicase activity. Reactions containing 1.2 nM WRN helicase, 0.5 nM forked duplex DNA substrate, and a specified compound from the NCI Diversity Set at a final concentration of 50 μM were incubated at 37 °C for 15 min, and reaction products subsequently were analyzed as described in *Materials and Methods*. Shown is a representative gel from a typical set of WRN helicase assays with a set of compounds. Arrow depicts a lane in which compound 5 strongly inhibits WRN helicase activity. ▲, heat-denatured DNA substrate control. (*B*) Graph representing percent control displacement values for 500 tested molecules from the NCI Diversity Set. Dashed line represents 70% inhibition of WRN helicase activity. (*C* and *D*) Effect of NSC 19630 on WRN helicase activity. Reactions containing 1.2 nM WRN and the indicated concentrations of NSC19630 were incubated with forked duplex DNA substrate at 37 °C for 15 min. A representative gel image is shown in *C*. Experiments were repeated three times, and percent control displacement values were determined. Error bars in *D* indicate SD. (*E*) Chemical structure and IC_{50} values for the selected small molecules from the NCI-DTP Diversity set.

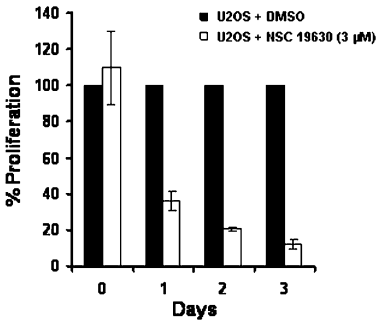

Fig. S2. Effect of NSC 19630 on U2OS cell proliferation. U2OS cells were treated with DMSO (control) or NSC 19630 (3 μM) for the indicated number of days. Percent proliferation was calculated as described in *Materials and Methods*. Cell proliferation then was determined with WST-1 reagent as described in *Materials and Methods*. Percent proliferation was calculated as the ratio of OD_{450} values obtained for U2OS cells grown in the presence of small-molecule inhibitor compared with cells grown in the presence of DMSO. Day 0 represents the effect of treatment on cell proliferation after 4 h. Cell proliferation data are the mean of at least two independent experiments with SDs indicated by error bars.

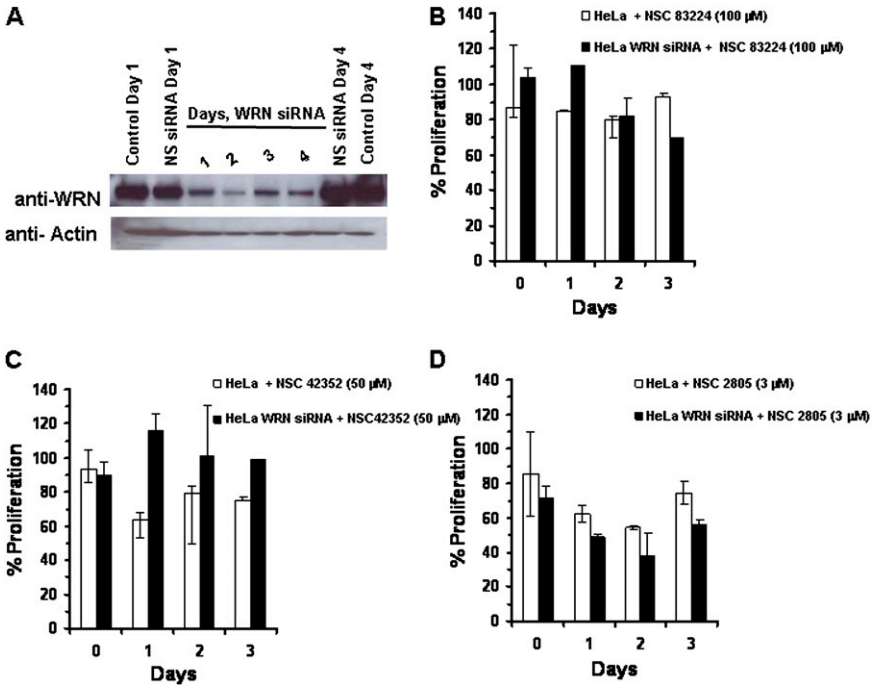

Fig. S3. Effect of NSC 83224, NSC 42352, and NSC 2805 on HeLa cell proliferation. HeLa cells were untransfected (control) or transfected with siRNA targeted specifically against WRN (WRN siRNA) for the indicated number of days as described in *SI Materials and Methods*. (*A*) Effect of WRN siRNA on WRN expression level in HeLa cells as determined by immunoblot analysis. Cell lysate (20 μg protein) was applied to each lane. The blot then was probed with anti-WRN antibody and as a loading control was reprobed with anti-actin antibody. (*B–D*) Cells were treated with DMSO or 100 μM NSC 83224 (*B*), 50 μM NSC 42352 (*C*), or 3 μM NSC 2805 (*D*) for the indicated number of days. Percent proliferation was calculated as described above. Day 0 represents the effect of treatment on cell proliferation after 4 h. Cell proliferation data are the mean of at least two independent experiments with SD indicated by error bars.

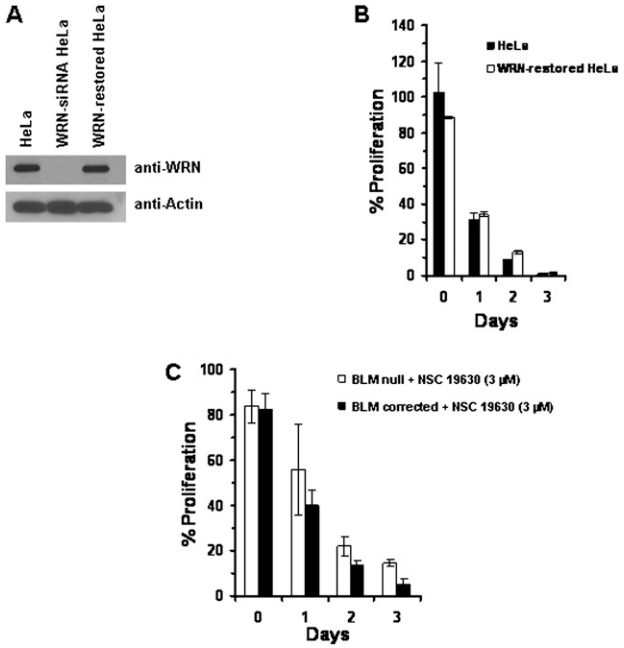

Fig. S4. Effect of NSC 19630 on cell proliferation is specific to WRN. (*A* and *B*) Recovery of WRN expression renders HeLa cells sensitive to NSC 19630. (*A*) After WRN siRNA transfection, cells were grown for 2 wk as described in *SI Materials and Methods*. Cell lysate protein (20 μg) was applied to each lane. The blot then was probed with anti-WRN antibody and as a loading control was reprobed with anti-actin antibody. As a control, equal amounts of cell lysate protein from HeLa and WRN-depleted HeLa cells were loaded on the gel. (*B*) After recovery of WRN expression, the specified cells were treated with DMSO or 3 μM NSC 19630 for the indicated number of days. Percent proliferation was calculated as described above. Day 0 represents the effect of treatment on cell proliferation after 4 h. Cell proliferation data are the mean of at least two independent experiments with SD indicated by error bars. (*C*) Effect of NSC 19630 on proliferation of BLM-null and BLM-corrected cells. $BLM^{-/-}$ (PSNG13) and $BLM^{+/+}$ (PSNF5) cells were treated with DMSO or with 3 μM NSC 19630 for the indicated number of days. Percent cell proliferation was determined as described above. Day 0 represents the effect of treatment on cell proliferation after 4 h. Cell proliferation data are the mean of at least two independent experiments with SD indicated by error bars.

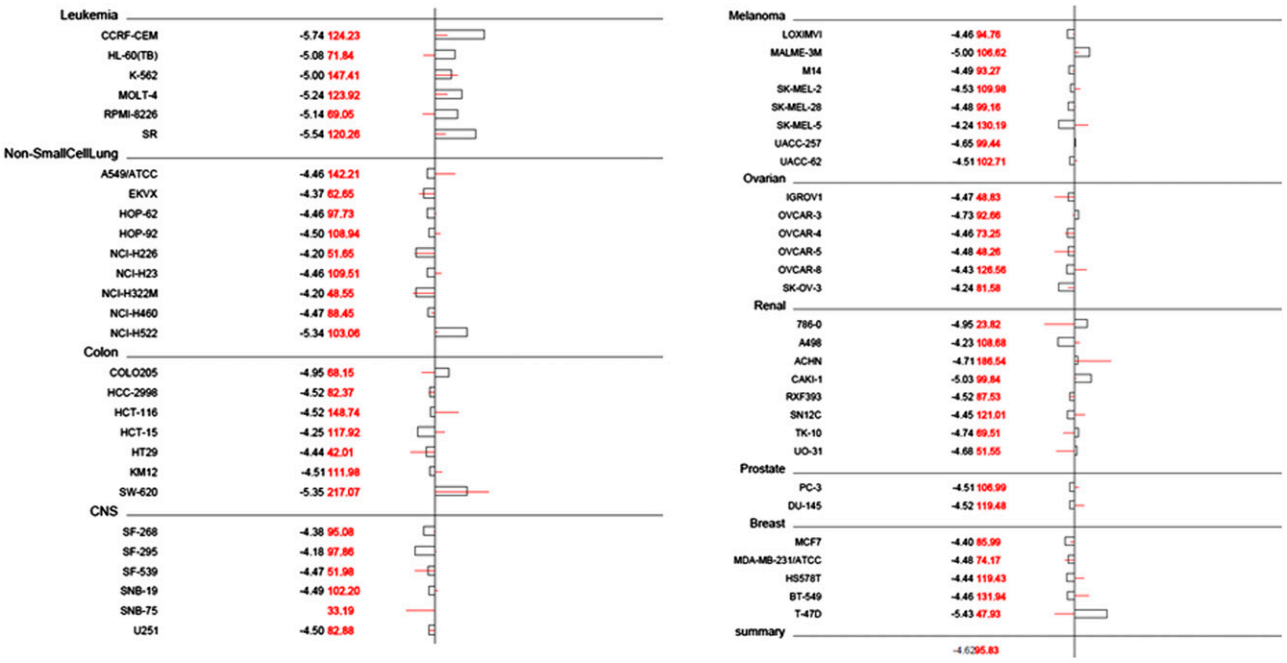

Fig. S5. Effect of NSC 19630 on proliferation of cancer and normal cell lines. WRN expression level does not correlate with the NSC 19630 sensitivity in the NCI60 screen. Mean graph representation of the WRN protein levels from cell lysates (red bars and numbers) and cellular sensitivity to NSC 19630 (black bars and numbers) in the NCI60 tumor cell line panel. For each parameter the overall average value for the NCI60 cell lines is represented by the vertical line (and is tabulated numerically at the bottom of the right panel). For WRN protein levels, the values are normalized relative to that observed in HeLa cells, taken as 100%. Drug sensitivity values are the negative log of the total growth inhibition parameter taken from the concentration–response curves. Bars projecting to the right of the central axis indicate cell lines showing higher-than-average protein levels or drug sensitivity. Bars projecting to the left indicate relatively resistant cell lines and lines showing less-than-average WRN protein levels.

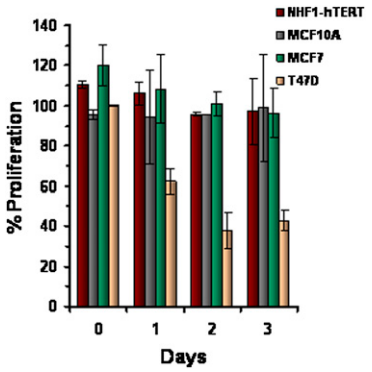

Fig. S6. Effect of NSC 19630 on proliferation of normal and breast cancer cell lines. Normal telomerase immortalized fibroblast (NHF1-hTERT), normal breast epithelium (MCF10A), and breast cancer cell lines (MCF7 and T47D) were treated with DMSO or 3 μM NSC 19630 for the indicated number of days. Percent cell proliferation was determined as described above. Day 0 represents the effect of treatment on cell proliferation after 4 h. Cell proliferation data are the mean of at least two independent experiments with SD indicated by error bars.

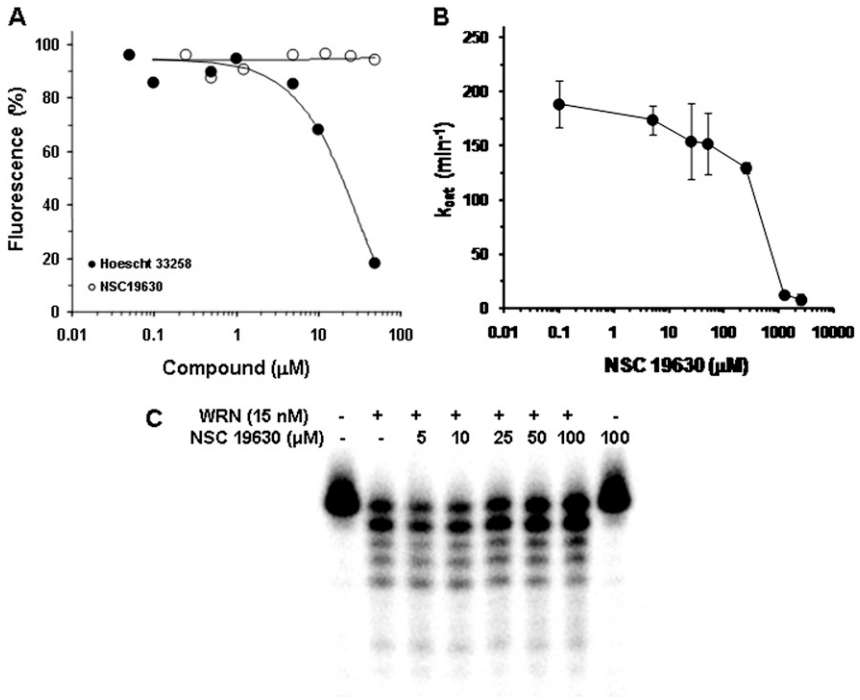

Fig. S7. Effect of NSC 19630 on Thiazole Orange DNA displacement and WRN ATPase and exonuclease activity. (*A*) The Thiazole Orange displacement assay was performed to determine if NSC 19630 binds the forked duplex DNA substrate used for helicase assays. Forked duplex DNA substrate (50 nM) was pre-incubated with Thiazole Orange (400 nM) and subsequently incubated with the indicated concentrations of NSC 19630 or Hoescht 33258 at 37 °C for 15 min as described in *SI Materials and Methods*. After incubation, fluorescence (excitation 485 nm, emission 520 nm) was measured using a FluoSTAR OPTIMA instrument. Percent fluorescence was calculated as the ratio of fluorescence readings obtained from DNA-binding incubations performed in the presence of NSC 19630 or Hoescht 33258 compared with incubations performed in the absence of either compound. Experiments were repeated two times. (*B*) Effect of NSC 19630 on WRN ATPase activity. Reactions containing 56 nM WRN and the indicated concentrations of NSC 19630 were incubated at 37 °C for 0–30 min and processed as described in *SI Materials and Methods*. Experiments were repeated three times, and k_{cat} values were determined with SD indicated by error bars. (*C*) Effect of NSC 19630 on WRN exonuclease activity. The indicated concentrations of NSC 19630 were incubated with WRN (15 nM) and a 5′ end-labeled duplex DNA substrate with a recessed 3′ end (0.5 nM) as described in *SI Materials and Methods*. WRN exonuclease reactions were initiated by the addition of WRN and incubation at 37 °C for 20 min. Reaction products were resolved on denaturing 14% polyacrylamide gels and imaged with a phosphorimager. A typical gel from at least three assays is shown.

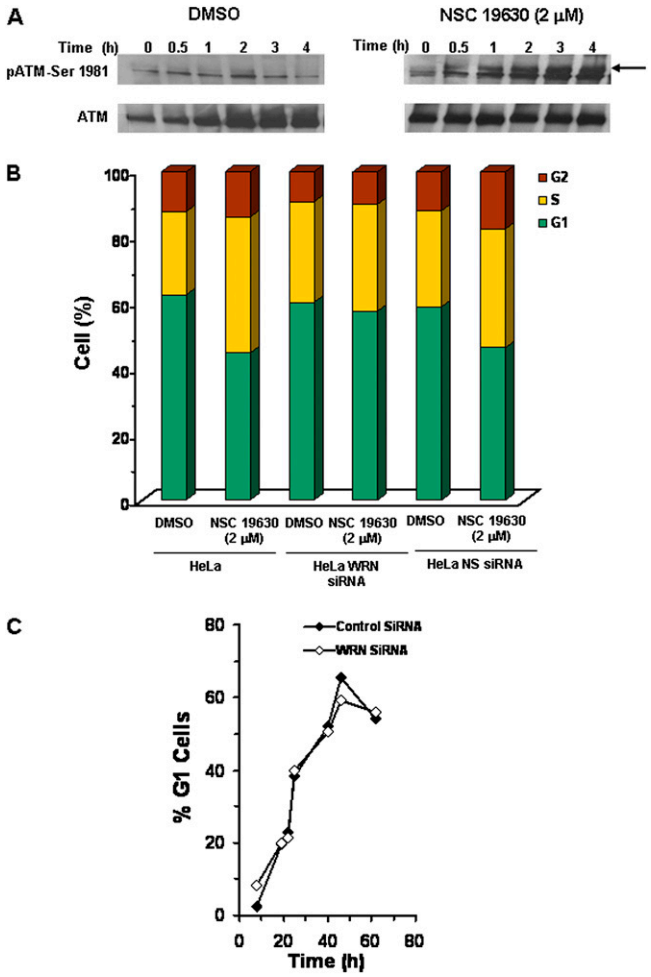

Fig. S8. Treatment of HeLa cells with NSC 19630 activates ATM and induces S-phase delay. (*A*) HeLa cells were treated with DMSO or 2 μM NSC 19630 for the indicated time intervals. Cell lysates then were prepared as described in *SI Materials and Methods* and analyzed by immunoblotting. Cell lysate protein (200 μg) was applied to each lane. The blot then was probed with anti-pATM Ser1981 antibody and as a control was reprobed with anti-ATM antibody. The arrow indicates the position of the phosphorylated ATM band. (*B* and *C*) Effect of NSC 19630 on cell cycle distribution. (*B*) HeLa cells were treated with 2 μM NSC 19630 or 1% DMSO as a control for 3 d. Cells then were prepared for flow cytometric analysis as described in *SI Materials and Methods*. The bar graph shows flow cytometric data for the DMSO- or NSC 19630-treated HeLa, control siRNA HeLa, or WRN siRNA-depleted HeLa cells. (*C*) Control siRNA HeLa cells or WRN siRNA-depleted HeLa cells were synchronized in S phase and analyzed by flow cytometry as described in *SI Materials and Methods*. Percentages of control siRNA or WRN siRNA-depleted HeLa cells in the G1 phase at the indicated time intervals were determined.

Table S1. Effect of selected NCI-DTP compounds on WRN helicase activity

NSC number	Unwinding (%)*
29192	20
19630	20
83224	15
2805	9
42352	19
48691	12
116533	2

*Indicates percent DNA duplex unwinding by helicase in presence of 50-μM compound compared with the DMSO control.

Table S2. Effect of NCI-DTP diversity set compounds on DNA unwinding by selected helicases

	Unwinding (%)*					
NSC number	RECQ1†	FANCJ†	BLM†	UvrD‡	DnaB‡	RecQ‡
29192	102	100	88	34	44	40
19630	98	102	97	97	121	99
83224	97	99	115	95	8	86
2805	99	103	100	91	110	100
42352	99	100	100	100	0	12
48961	85	100	86	61	0	46
116533	62	0	98	35	76	4

*Indicates percent DNA duplex unwinding by helicase in presence of 50-μM compound compared with the DMSO control.
†Purified human recombinant protein.
‡Purified *Escherichia coli* recombinant protein.