Werner Syndrome Helicase Has a Critical Role in DNA Damage Responses in the Absence of a Functional Fanconi Anemia Pathway

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Abstract

Werner syndrome is genetically linked to mutations in WRN that encodes a DNA helicase-nuclease believed to operate at stalled replication forks. Using a newly identified small-molecule inhibitor of WRN helicase (NSC 617145), we investigated the role of WRN in the interstrand cross-link (ICL) response in cells derived from patients with Fanconi anemia, a hereditary disorder characterized by bone marrow failure and cancer. In FA-D2−/− cells, NSC 617145 acted synergistically with very low concentrations of mitomycin C to inhibit proliferation in a WRN-dependent manner and induce double-strand breaks (DSB) and chromosomal abnormalities. Under these conditions, ataxia–telangiectasia mutated activation and accumulation of DNA-dependent protein kinase, catalytic subunit ps2056 foci suggested an increased number of DSBs processed by nonhomologous end-joining (NHEJ). Rad51 foci were also elevated in FA-D2−/− cells exposed to NSC 617145 and mitomycin C, suggesting that WRN helicase inhibition interferes with later steps of homologous recombination at ICL-induced DSBs. Thus, when the Fanconi anemia pathway is defective, WRN helicase inhibition perturbs the normal ICL response, leading to NHEJ activation. Potential implication for treatment of Fanconi anemia–deficient tumors by their sensitization to DNA cross-linking agents is discussed. Cancer Res; 73(17); 5497–507. ©2013 AACR.

Introduction

Werner syndrome, characterized by premature aging and cancer predisposition (1), is caused by autosomal recessive mutations in WRN (2), encoding a RecQ DNA helicase-nuclease implicated in genomic stability (3, 4). WRN helicase can unwind key homologous recombination DNA intermediates and interacts with proteins implicated in DNA replication, recombinational repair, and telomere maintenance (5). Werner syndrome cells are sensitive to certain DNA-damaging agents, and display defects in resolution of recombination intermediates and a prolonged S-phase (6). WRN is required for normal replication fork progression after DNA damage or fork arrest (7).

Werner syndrome cells are sensitive to interstrand cross-link (ICL)–inducing agents (8) and undergo apoptosis in S-phase (9). Cellular studies suggest WRN helicase, in conjunction with BRCA1, is required to process DNA ICLs (10). WRN is required for ataxia–telangiectasia mutated (ATM) activation and the intra-S-phase checkpoint in response to ICL-induced double-strand breaks (DSB; ref. 11), suggesting WRN and ATM collaborate in response to collapsed forks at ICL-induced DSBs. Biochemical studies using a reconstituted system with a DNA substrate harboring a psoralen ICL suggested that WRN helicase–dependent and 53BP1 or Ku exacerbates genomic instability in FANC D2−/− cells (12).

Patients with Fanconi anemia suffer from progressive bone marrow failure and cancer predisposition, and their cells (such as Werner syndrome cells) are sensitive to ICL agents, only to a greater extent (13). In fact, a chromosome breakage test using Fanconi anemia patient cells exposed to an ICL-inducing agent is used as a primary diagnostic tool for Fanconi anemia (14). The Fanconi anemia pathway is involved in initial recognition and unhooking of an ICL and subsequent repair of the ICL-induced DSB in conjunction with homologous recombination or translesion synthesis pathways, but the detailed molecular mechanism is an active area of investigation (15). One function of the Fanconi anemia pathway is to channel DSBs through the homologous recombination pathway, thereby preventing inappropriate engagement of breaks by error-prone nonhomologous end-joining (NHEJ; refs. 16, 17). However, the relationship between DNA repair pathways is complex, as evidenced by a recent mouse study showing that deletion of 53BP1 or Ku exacerbates genomic instability in FANC D2−/− cells (18).
To understand the role of WRN in the ICL response, we examined effects of a small-molecule WRN helicase inhibitor in a Fanconi anemia–mutant genetic background. A more potent structural analog of the previously identified parent compound NSC 19630 (19) was discovered that is blocked from potential thiol reactivity but retains the ability to modulate WRN function in vivo. The WRN helicase inhibitor NSC 617145 acted synergistically with a very limited concentration of mitomycin C to induce DSB accumulation and chromosomal abnormalities, and activate the DNA damage response in Fanconi anemia–mutant cells. NSC 617145 exposure resulted in enhanced accumulation of DNA-dependent protein kinase, catalytic subunit (DNA-PKcs) p52056 foci and Rad51 foci in mitomycin C–treated Fanconi anemia–deficient cells, suggesting that WRN helicase inhibition prevents processing of Rad51-mediated recombination products and activates NHEJ. WRN helicase may be a suitable target for chemotherapeutic strategies in Fanconi anemia–deficient tumors.

Materials and Methods

Cell lines and culture

HeLa (CCL-2), U2OS (HTB96), and HCT116 (CCL-247) cell lines were obtained from American Type Culture Collection where they were tested and authenticated. HCT116 p53 double knockout cells were obtained from the laboratory of Dr. B. Vogelstein (Johns Hopkins University, Baltimore, MD) where they were tested and authenticated (20). These cell lines were grown in Dulbecco’s Modified Eagle Medium supplemented with 10% FBS, 1% penicillin–streptomycin, and 1% t-glutamine at 37°C in 5% CO2. PSN5 [Bloom syndrome (BLM)–corrected] and PSNG13 (BLM-mutant) are isogenic cell lines that were obtained from the laboratory of Dr. I. Hickson (University of Copenhagen, Copenhagen, Denmark) where they were tested and authenticated (21, 22). PSN5 is a stable BLM cell line expressing a FLAG epitope-tagged, wild-type BLM protein from the cytomegalovirus (CMV) promoter in a pcDNA3-based construct. PSNG13 is a control BLM cell transfected containing the pcDNA3 vector only. The BLM-mutant and -corrected cell lines were grown in the same media as HeLa cells but supplemented with 350 μg/mL G418. The simian virus 40–transformed Fanconi anemia–mutant fibroblasts, PD20 (FA-D2), GM6914 (FA-A), and their respective corrected counterparts were provided by Fanconi Anemia Cell Repository at Oregon Health & Science University (Portland, OR) where they were tested and authenticated. Fanconi anemia–mutant and -corrected cells were grown in the same medium as HeLa cells but supplemented with 0.2 ng/mL puromycin.

Cell proliferation assays

Proliferation was measured using WST-1 assay (Roche) as described previously (19).

siRNA transfection and Western blot analysis

WRN siRNA (WH: 5′-UAGAGGGAACUUUCGCAAUU-3′ and/or CG: 5′-GUGUAUAGUACGAGUCAGUGAU-3′) and control siRNA (19) were transfected using Lipofectamine 2000 as per the manufacturer’s protocol (Invitrogen). Cells were plated to 50% to 60% confluence in 10-cm dishes 24 hours 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 were subsequently incubated for 6 hours. After 24 hours, a second transfection was carried out similarly. Seventy-two hours after the initial transfection, cells were harvested for preparing lysate or treated with small-molecule compounds or dimethyl sulfoxide (DMSO) at the indicated concentrations and cell proliferation was measured using WST-1 reagent (Roche) as described for HeLa cells. For Fanconi anemia–mutant cells, siRNA transfection and treatment with NSC 617145 was carried out as described previously except that OD_{450} was measured after 48 hours of treatment with mitomycin C and/or NSC 617145.

For lysate preparation, cells were washed twice with 1× PBS. Radioimmunoprecipitation assay (RIPA) buffer [10 mmol/L sodium phosphate (pH 7.2), 300 mmol/L NaCl, 0.1% SDS, 1% NP-40, 1% sodium deoxycholate, and 2 mmol/L EDTA] was added to the cells and the cells were incubated at 4°C for 30 minutes. Cells were scraped and the suspension was further incubated on ice for 30 minutes. Cell suspension was centrifuged at 18,500 × g for 10 minutes at 4°C and supernatant was collected. Twenty microgram of the lysate was loaded on 8% to 16% SDS-PAGE. Protein was transferred onto a polyvinylidene difluoride (PVDF) membrane and blot was probed with anti-WRN mouse monoclonal antibody (1:1,000; Spring Valley Laboratories). For secondary antibody, peroxidase-conjugated anti-mouse immunoglobulin G (IgG; 1:1,000; Vector Laboratories) was used. Blot was developed using ECL Plus Western Blot Detection Kit as per the manufacturer’s protocol (Amersham). As a loading control, blot was stripped and then reprobed with anti-actin antibody (1:5,000; Sigma).

Analysis of metaphase chromosomes

Cell harvest and metaphase slide preparation was carried out for metaphase analysis as described previously (23). See Supplementary Data for additional information.

Results

Inhibition of WRN helicase activity by small-molecule NSC 617145

To identify small molecules that inhibit WRN helicase activity in a more potent manner than the previously identified compound NSC 19630 (19), we selected four close structural analogs and three compounds whose pattern of activity in the NCI60 screen matched NSC 19630 for analysis (Supplementary Fig. S1). One of the latter compounds, NSC 617145, is also a structural analog but blocked with respect to thiol reactivity in the five-membered rings by Cl atoms. NSC 617145 inhibited WRN helicase activity in a concentration-dependent manner in vitro (Supplementary Fig. S2A), yielding an IC_{50} value of 230 mmol/L. NSC 617145 inhibited WRN ATPase in a dose-dependent manner (Supplementary Fig. S2B). No detectable effect on WRN exonuclease was observed (Supplementary Fig. S2C).

To examine specificity of WRN helicase inhibition, we tested 5 mmol/L NSC 617145 (~20-fold >IC_{50} value) on DNA unwinding catalyzed by various helicases. No significant inhibition of unwinding was observed with BLM, FANCJ, ChlR1, RecQ, and...
UvrD, and only a very modest (7%) inhibition of RECQ1 (Supplementary Fig. S2D) was observed.

To determine their potency in vivo, HeLa cells were exposed to increasing concentrations of selected analogs or DMSO for 0 to 3 days. Of the selected analogs, NSC 617145 showed maximal inhibition of proliferation (98%) at the lowest concentration of NSC 617145 (1.5 \( \mu \text{mol/L} \); Fig. 1A; Supplementary Fig. S3). To address whether the effect was cytostatic or cytotoxic, HeLa cells were exposed to NSC 617145 for 3 days, replenished with media lacking NSC617145, and allowed to recover for 0 to 3 days (Fig. 1B). The lack of recovery from NSC 617145 suggested a cytotoxic effect. To determine whether the effect of NSC 617145 was WRN-dependent, we compared its effect on HeLa cells depleted of WRN (≥90%) with NS-siRNA–transfected cells. WRN-depleted cells grown in the presence of 1.5 \( \mu \text{mol/L} \) NSC 617145 were resistant to its antiproliferative effects, whereas NS-siRNA HeLa cells were exposed to NSC 617145 for 3 days, replenished with media lacking NSC 617145, and allowed to recover for 0 to 3 days (Fig. 1B). The lack of recovery from NSC 617145 suggested a cytotoxic effect. To determine whether the effect of NSC 617145 was WRN-dependent, we compared its effect on HeLa cells depleted of WRN (≥90%) with NS-siRNA–transfected cells. WRN-depleted cells grown in the presence of 1.5 \( \mu \text{mol/L} \) NSC 617145 were resistant to its antiproliferative effects, whereas NS-siRNA HeLa cells were highly sensitive to NSC 617145 (Fig. 1C). These results were confirmed by 2 different WRN-siRNA molecules that independently conferred resistance to NSC 617145 (Supplementary Fig. S4). Western blot analyses showed that WRN depletion by either WRN-siRNA or combination was more than 90% (Supplementary Fig. S4).

NSC 617145 may exert its effect through a mechanism in which the NSC 617145–inhibited WRN helicase induces greater damage by interfering with a compensatory mechanism(s). To determine whether the effect of NSC 617145 is dependent on a helicase-active version of WRN, HeLa cells were depleted of endogenous WRN, and siRNA-resistant WRN [wild-type or helicase inactive K577M (24)] was expressed. The resulting cells were tested for sensitivity to NSC 617145. It was observed that the WRN-depleted cells rescued with wild-type WRN were sensitive to the WRN helicase inhibitor compared with empty vector–transfected cells, supporting the finding that the inhibition of cell proliferation by NSC 617145 is WRN-dependent (Supplementary Fig. S5). However, WRN-depleted cells expressing the helicase-inactive WRN-K577M behaved similar to the WRN-depleted cells transfected with empty vector, suggesting that they were resistant to the negative effect of WRN helicase inhibitor NSC 617145 on cell viability.

Because NSC 617145 inhibited proliferation of p53-inactivated HeLa cells, we examined its effect on proliferation of U2OS cells with wild-type p53. NSC 617145 (1.5 \( \mu \text{mol/L} \)) inhibited U2OS proliferation by 80% after day 2 (Supplementary Fig. S6A), suggesting that the effect of NSC 617145 was not dependent on p53 status. We also tested a pair of isogenic p53-negative and -positive HCT116 cells for sensitivity to NSC 617145 (1.5 \( \mu \text{mol/L} \)). Both cell lines were sensitive to the compound; however, p53-deficient cells were 2-fold more resistant than p53-proficient cells (Supplementary Fig. S6B). Because NSC 617145 exerted a WRN-dependent effect on proliferation, we evaluated whether cells mutated for the sequence-related BLM helicase were sensitive. BLM-null and -corrected cells displayed similar sensitivity to NSC 617145 (Supplementary Fig. S6C), suggesting BLM does not play a role.

Figure 1. NSC 617145 inhibits cell proliferation in a WRN-specific manner. A, HeLa cells were treated with DMSO or indicated NSC 617145 concentration for 0 to 3 days. B, HeLa cells were exposed to 1.5 \( \mu \text{mol/L} \) NSC 617145 for 3 days, replenished with media lacking NSC 617145, and allowed to recover for 0 to 3 days. C, HeLa cells either untransfected (HeLa) or transfected with nonspecific siRNA (NS siRNA) or WRN-specific siRNA (WRN siRNA) were treated with DMSO or 1.5 \( \mu \text{mol/L} \) NSC 617145 for indicated number of days. Cell proliferation was then determined with WST-1 reagent. Percentage proliferation was calculated. Experiments were repeated three times, and error bars indicate SD. This applies to all figures.
**Cellular exposure to NSC 617145 causes accumulation of DSBs, blocked replication forks, and apoptosis**

Because WRN-deficient cells are defective in resolution of recombination intermediates (6), NSC 617145 inhibition of WRN helicase activity might result in DSBs at blocked replication forks. Therefore, we analyzed the effect of NSC 617145 on γ-H2AX foci, a marker of DSBs. Exposure of HeLa cells to 0.25 μmol/L NSC 617145 elevated γ-H2AX foci approximately 18-fold compared with DMSO-treated cells (Supplementary Fig. S7A and S7B). WRN-siRNA–treated HeLa cells showed a similar low number of γ-H2AX foci in NSC 617145– and DMSO-treated cells, suggesting that inhibition of WRN activity by NSC 617145 led to DSB accumulation in a WRN-dependent manner. H2AX phosphorylation can be induced by a wide range of phenomena including DSBs; therefore, we examined the effect of NSC 617145 on 53BP1 foci, an independent marker of DNA damage (25). Cellular exposure to 0.25 μmol/L NSC 617145 elevated 53BP1 foci 3.5-fold compared with DMSO-treated cells, confirming NSC 617145 induced DNA damage (Supplementary Fig. S7C and S7D).

HeLa cells exposed to 1.5 μmol/L NSC 617145 showed a 4-fold increase in apoptosis as compared with DMSO-treated cells (Supplementary Fig. S8). WRN-depleted HeLa cells showed no difference in level of apoptosis in NSC 617145- and DMSO-treated cells, showing WRN-dependent induction of apoptosis by NSC 617145. Failure to repair DNA damage would affect replication fork progression; therefore, we determined the effect of NSC 617145 on proliferating cell nuclear antigen (PCNA) foci formation. HeLa cells exposed to 0.25 μmol/L NSC 617145 showed an elevated number of PCNA foci (~22-fold) compared with DMSO-treated cells (Supplementary Fig. S9A and S9B). In contrast, WRN-depleted cells showed similar levels of PCNA staining for NSC 617145– or DMSO-treated cells, suggesting WRN-dependent accumulation of stalled replication foci. Consistent with the PCNA induction, exposure of HeLa cells to NSC 617145 (1 μmol/L) resulted in an increased population in S-phase compared with DMSO-treated cells (Supplementary Fig. S10). We also observed from fluorescence-activated cell sorting (FACS) analysis a sub-G1 fraction of NSC 617145–treated HeLa cells (data not shown), consistent with the induction of apoptosis by the WRN helicase inhibitor. WRN-depleted cells exposed to 1 μmol/L NSC 617145 showed a similar percentage of cells in S-phase compared with DMSO-treated cells, suggesting that NSC 617145 induced prolonged S-phase in a WRN-dependent manner.

**NSC 617145 induces WRN binding to chromatin and proteasomal degradation**

We reasoned that NSC 617145 might target cellular WRN for helicase inhibition and induce toxic DNA lesions mediated by WRN’s interaction with genomic DNA, prompting us to ask whether poisoned WRN became enriched in the chromatin fraction. Western blot analysis of nuclear soluble and chromatin-bound fractions prepared from HeLa cells exposed to NSC 617145 showed a dose-dependent increase in WRN bound to chromatin (Fig. 2A). We also observed that the amount of WRN from extract of HeLa cells exposed to NSC 617145 (0.75 μmol/L) for 6 hours was reduced compared with DMSO-treated cells (Fig. 2B). Inclusion of proteasome inhibitor MG132 restored WRN level to that of DMSO-treated cells (Fig. 2B). Thus, NSC 617145 causes WRN to become degraded by a proteasome-mediated pathway. Consistent with reduction of WRN protein in NSC 617145–treated cells, helicase activity catalyzed by immunoprecipitated WRN from an equivalent amount of total extract protein from HeLa cells treated with NSC 617145 (0.75 μmol/L; 4 hours) was reduced 4-fold compared with helicase activity by immunoprecipitated WRN from DMSO-treated cells (Fig. 2C).

**Synergistic effect of NSC 617145 and DNA cross-linking agent mitomycin C on cell proliferation**

We sought to use the newly identified WRN helicase inhibitor as a tool to explore whether WRN helicase activity helps cells cope with stress imposed by the DNA cross-linking agent mitomycin C. Treatment with either NSC 617145 (0.5 μmol/L) or mitomycin C (9.4 nmol/L) exerted no significant effect on proliferation. However, cotreatment with both NSC 617145 (0.5 μmol/L) and mitomycin C (9.4 nmol/L) resulted in a 45% reduction in proliferation (Fig. 3A). In contrast, treatment with NSC 617145 (0.5 μmol/L) and hydroxyurea (0–5 mmol/L) had a similar effect on proliferation compared with hydroxyurea alone (Supplementary Fig. S11A), suggesting that the apparent synergism between NSC 617145 and mitomycin C is not a general effect imposed by other forms of replicative stress such as hydroxyurea that depletes the nucleotide pool.

**NSC 617145–treated Fanconi anemia–mutant cells are hypersensitive to mitomycin C**

On the basis of the synergistic effect of NSC 617145 and mitomycin C, we hypothesized that WRN might participate in a pathway that is partially redundant with Fanconi anemia–mediated ICL repair. For this, we examined sensitivity of FA-D2–mutant and -corrected cells to mitomycin C and NSC 617145. Dose–response curves for mitomycin C and NSC 617145 sensitivity of FA-D2 cells indicated drug concentrations at which only modest effects on proliferation were observed (Supplementary Fig. S11B and S11C). NSC 617145 (0.125 μmol/L) or mitomycin C (9.4 nmol/L) exerted only a mild effect on cell proliferation; however, a significant 45% reduction was observed for cells treated with both NSC 617145 and mitomycin C (Fig. 3B). In contrast, FA-D2+/+ cells showed only a 10% reduction of proliferation by combined treatment of NSC 617145 and mitomycin C (Fig. 3B). Sensitization of FA-D2−/− cells to mitomycin C by WRN helicase inhibition is distinct from the effect imposed by a deficiency or inhibition of proteins in the NHEJ pathway (LIG4 and KU70), which suppress ICL-sensitivity of Fanconi anemia–deficient cells (16, 17).

Combinatorial treatment of FA-A−/− cells with NSC 617145 (0.125 μmol/L) and mitomycin C (9.4 nmol/L) resulted in 60% reduction in proliferation compared with very modest effects exerted by either agent alone (Fig. 3C). Moreover, the effect was dependent on FANCA status as only 10% reduction in proliferation was observed for FA-A−/− cells exposed to both mitomycin C and NSC 617145. Thus, NSC 617145 acts in a
synergistic manner with mitomycin C in either the FANCA- or FANCD2-mutant background.

Treatment with both NSC 617145 (0.125 μmol/L) and hydroxyurea (0–1.25 mmol/L) resulted in a very similar effect on proliferation as compared with hydroxyurea alone in both FA-D2−/− and FA-D2+/− cells (Supplementary Fig. S11D and S11E). Pretreatment of FA-D2−/− cells with hydroxyurea for 24 hours followed by exposure to 0.125 μmol/L NSC 617145 for 48 hours also showed very similar inhibition of proliferation compared with cells only exposed to hydroxyurea (Supplementary Fig. S11F), indicating NSC 617145 does not sensitize cells to hydroxyurea.

NSC 617145 causes FA-D2−/− mutant cells to be hypersensitive to mitomycin C in a WRN-dependent manner

To determine whether enhanced mitomycin C sensitivity of FA-D2−/− cells was mediated through inhibition of WRN function by NSC 617145, WRN was depleted by RNA interference in FA-D2−/− or FA-D2+/− cells by 90% or more compared with NS-siRNA cells (data not shown). WRN-depleted FA-D2−/− cells grown in the presence of 0.125 μmol/L NSC 617145 and 9.4 mmol/L mitomycin C were resistant to the antiproliferative effects of combined treatment (Fig. 3D), whereas NS-siRNA FA-D2−/− cells were sensitive to cotreatment with NSC 617145 and mitomycin C, as evidenced by 40% reduction in proliferation (Fig. 3E). In contrast, no difference in proliferation was observed with WRN-depleted or NS-siRNA-treated FA-D2+/+ cells cotreated with these NSC 617145 and mitomycin C concentrations (Fig. 3D and E). Furthermore, WRN depletion in FA-D2−/− cells did not affect mitomycin C sensitivity as compared with NS-siRNA–treated or untransfected cells (Supplementary Fig. S12), suggesting WRN helicase inhibition by NSC 617145 interfered with the ability of FA-D2−/− cells to cope with mitomycin C–induced DNA damage.

NSC 617145 and mitomycin C act synergistically to induce DNA damage and activate ATM

Failure to repair DNA ICLs in dividing cells would be expected to result in blocked replication forks and hence DSBs. Cotreatment of FA-D2−/− cells with NSC 617145 (0.125 μmol/L) and mitomycin C (9.4 mmol/L) resulted in a substantial increase in percentage (80%) of cells with more than 15 γ-H2AX foci (Fig. 4A). In contrast, only 30% FA-D2+/− cells exposed to NSC 617145 and mitomycin C showed more than 15 γ-H2AX foci (Fig. 4B). The difference in sensitivity of FA-D2−/− versus FA-D2+/− cells exposed to very low concentrations of either mitomycin C or WRN inhibitor was modest (NSC 617145) or hardly apparent (mitomycin C) compared...
with the cotreatment. Thus, accumulation of mitomycin C–
induced DNA damage in FA-D2−/− cells is increased by NSC 617145.

Exposure of FA-D2−/− cells with a dose range (0.125–1.5 
μmol/L) of NSC 617145 led to activation of ATM as detected by
pATM-Ser1981 at 0.5 μmol/L NSC 617145 (Supplementary
Fig. S13); however, no significant activation of ATM
was observed at lower doses. In FA-D2+/− cells, no appreci-
able ATM activation was detected except at the highest NSC 617145
concentration tested, 1.5 μmol/L. Upon coexposure of 
FA-D2−/− cells with a very low dose of NSC 617145 (0.125 
μmol/L) and mitomycin C (9.4 nmol/L), there was a signif-
icant accumulation of pATM-Ser1981, whereas ATM
activation was not detected in FA-D2+/− cells (Fig. 5A).
FA-D2−/− cells exposed to very low dose of NSC 617145 (0.125 
μmol/L) and mitomycin C (9.4 nmol/L) retained high
levels of pATM-Ser1981 even after 24-hour exposure (data
not shown), suggesting a significant delay in damage repair
when WRN helicase was inhibited. Thus, NSC 617145 and
mitomycin C act synergistically to induce DNA damage and
activate ATM.

**NSC 617145 elevated mitomycin C–induced chromosomal instability and induced accumulation of DNA-PKcs pS2056 foci in FA-D2−/−mutant cells**

Recent studies suggest the Fanconi anemia pathway plays an
important role in preventing aberrant DNA repair (16, 17).
Because NHEJ factors have high affinity for DNA ends (26), the
accumulated DSBs in FA-D2−/− cells, when WRN helicase
activity is pharmacologically inhibited, might promote gen-
ic instability when captured by error-prone pathways. To
address this, we examined metaphase spreads from FA-D2−/−
and FA-D2+/− cells for chromosomal aberrations (Fig. 5B; ref.
27). FA-D2−/− cells exposed to NSC 617145 (0.125 μmol/L) and
mitomycin C (9.4 nmol/L) showed a 4-fold increase in abnor-
mal chromosome structures compared with either agent alone
(Fig. 5C). Enhanced chromosomal instability was dependent
on FANCD2 status as evidenced by the relatively low level of
chromosomal breaks in FA-D2+/− cells exposed to NSC 617145
and/or mitomycin C.

DNA-dependent protein kinase complex is required for 
NHEJ in conjunction with Ku70/80 and XRCC4/Ligase IV;
moreover, DNA-PKcs pS2056 is detected at DSBs (28). To
substantiate the role of NHEJ in DSB processing when WRN
helicase is inhibited, FA-D2−/− cells were examined for DNA-
PKcs pS2056 foci formation. Cotreatment of FA-D2−/−
cells with limited concentrations of NSC 617145 (0.125 μmol/L) and
mitomycin C (9.4 nmol/L) resulted in 70% of cells with more
than 15 DNA-PKcs pS2056 foci (Fig. 6A and B). In contrast,
only 15% of FA-D2+/− cells exposed to NSC 617145 and mitomycin C
showed more than 15 DNA-PKcs pS2056 foci (Fig. 6A and B).
The difference in sensitivity between FA-D2−/− and FA-D2+/− cells
exposed to very low concentrations of either mitomycin C
or NSC 617145 was modest (mitomycin C) or hardly apparent
(NSC 617145) compared with cotreatment, suggesting that
when WRN helicase activity is inhibited, FA-D2−/− cells show

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**Figure 3.** NSC 617145 exposure enhances sensitivity of Fanconi anemia–mutant cells to mitomycin C in a WRN-dependent manner. A, HeLa cells were treated with NSC 617145 (0.5 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 3 days. B, FA-D2−/− and FA-D2+/− cells were treated with NSC 617145 (0.125 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 2 days. C, FA-A−/− and FA-A+/+ cells were treated with NSC 617145 (0.75 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 2 days. D, FA-D2−/− and FA-D2+/− cell lines transfected with WRN siRNA were treated with NSC 617145 (0.125 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 2 days. E, FA-D2−/− and FA-D2+/+ cell lines transfected with NS siRNA were treated with NSC 617145 (0.125 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 2 days. Cell proliferation was determined. Percentage proliferation was calculated.

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increased chromosomal instability due to more DSBs repaired by the error-prone pathway. Strikingly, FA-D2+/+ cells cotreated with NSC 617145 and mitomycin C, A, FA-D2−/− and FA-D2+/+ cells were treated with NSC 617145 (0.125 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 2 days. Cells were stained with anti-γH2AX antibody or 4',6-diamidino-2-phenylindole (DAPI) and imaged. B, percentage of cells with γH2AX foci (≤15 or >15, as indicated).

**NSC 617145 exposure results in accumulation of Rad51 foci in FA-D2−/− cells**

WRN plays a physiologic role in resolution of Rad51-dependent homologous recombination products (6). Expression of dominant-negative Rad51 or bacterial resolvase protein RusA could suppress homologous recombination or lead to the generation of recombinants, respectively, resulting in improved survival of WRN−/− cells. Because Rad51 foci are formed at sites of ICL lesions independently of FA-D2 status (29), we reasoned that WRN helicase inhibition in mitomycin C–treated FA-D2−/− cells might result in accumulation of homologous recombination intermediates. Therefore, we investigated the effect of NSC 617145 and mitomycin C treatment on Rad51 foci formation in Fanconi anemia–mutant and -corrected cells. We did not observe any significant difference in percentage of cells that displayed Rad51 foci between FA-D2−/− and FA-D2+/+ upon cellular exposure with mitomycin C (9.4 nmol/L), as reported previously at higher doses of mitomycin C (29) or NSC 617145 (0.125 μmol/L; Fig. 7A and B). In both cell lines, approximately 50% showed Rad51 foci, similar to that observed for DMSO-treated cells. Cotreatment of FA-D2−/− cells with NSC 617145 and mitomycin C resulted in 90% of cells staining positive for Rad51 foci. In contrast, FA-D2+/+ cells exposed to both agents showed a percentage of cells with Rad51 foci very similar to DMSO-treated cells (Fig. 7A and B). Collectively, these results suggest that mitomycin C–induced DNA cross-links in FA-D2−/− cells exposed to WRN helicase inhibitor are converted to DSBs and the homologous recombination pathway is activated but homologous recombination intermediates fail to be subsequently resolved.

**Discussion**

In this study, we discovered a new inhibitor of WRN helicase activity (NSC 617145) that negatively affects cell proliferation and induces DNA damage in a WRN-dependent manner. WRN depletion negates biologic activity of NSC 617145, suggesting targeted WRN helicase inhibition interferes with...
normal cellular DNA metabolism. The synergistic effect of NSC 617145 and mitomycin C was not observed for hydroxyurea, suggesting that impairment of WRN helicase activity exacerbates the effect of mitomycin C–induced DNA damage during replication but not an effect exerted by an agent (hydroxyurea) that primarily induces replication stress. We used the WRN helicase inhibitor to sensitize a Fanconi anemia–mutant because mounting evidence points toward a pivotal role of the Fanconi anemia pathway to coordinate a robust ICL response when WRN is also likely to act. WRN helicase inhibition by NSC 617145 makes FA-A or FA-D2 cells highly sensitive to very low mitomycin C concentrations that would otherwise be only marginally active in normal cells. Furthermore, the effect of combined mitomycin C/NSC 617145 treatment is a consequence of a Fanconi anemia pathway deficiency rather than a defect in a specific Fanconi anemia gene.

The synergistic effect of NSC 617145 and mitomycin C in the FA-D2–mutant was apparent by robust γ-H2AX staining, ATM activation, and increased chromosomal abnormalities. Moreover, accumulation of DNA-PKcs pS2056 foci suggests that FA-D2 cells attempted to deal with mitomycin C–induced DSBs by error-prone NHEJ when WRN helicase was pharmacologically inhibited. This finding builds on previous evidence that the Fanconi anemia pathway plays an important role in preventing aberrant NHEJ-mediated repair (16, 17), and implicates a role of WRN helicase in repair pathway choice. In addition to its helicase-dependent role in recombinational repair, WRN may have a structural role to enable repair of mitomycin C–induced DNA damage. This would be akin to the observation that a WRN helicase/exonuclease double-mutant complemented the NHEJ defect of WRN–/– cells (30). Although experimental data suggest that WRN participates in an alternative NHEJ repair pathway of DSBs induced by increased

**Figure 5.** FA-D2–mutant cells cotreated with NSC 617145 and mitomycin C display ATM activation and increased chromosomal instability. A, FA-D2–/– and FA-D2+/+ cells were cotreated with 0.125 μmol/L NSC 617145 and 9.4 nmol/L mitomycin C for 3 hours. Cell lysates were prepared and analyzed by immunoblotting by using anti-pATM Ser1981 antibody. As a control, blot was reprobed with anti-ATM antibody. Arrow indicates phosphorylated ATM. B, FA-D2+/+ and FA-D2–/– cells were treated with NSC 617145 (0.125 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 2 days. Chromosome spreads were analyzed. Arrow indicates chromatid break, chromatid loss, or radial. Each radial is counted as equivalent to two chromatid breaks. C, chromatid breaks per metaphase.
reactive oxygen species in chronic myeloid leukemia cells (31), it is unclear whether this pathway is relevant to a role of WRN helicase to confer mitomycin C resistance in Fanconi anemia–deficient fibroblasts. Further studies will be necessary to evaluate whether an alternate end-joining pathway or homologous recombination is important for repair of mitomycin C–induced lesions in the WRN helicase-inhibited condition.

We also observed Rad51 foci accumulation in FA-D2+/+/FA-D2−/− cells cotreated with mitomycin C and NSC 617145, suggesting that homologous recombination was activated and at least some DSBs were channeled into homology-mediated repair. However, as WRN plays a role in resolution of homologous recombination intermediates (6), NSC 617145 inhibition of WRN helicase activity might lead to accumulation of homologous recombination structures. We observed phosphorylation of DNA-PKcs only under conditions of WRN helicase inhibition in FA-D2−/− cells, suggesting that NHEJ was elicited; however, the mechanism of DNA-PKcs recruitment is still unclear.

Development of helicase inhibitors such as NSC 617145 that possess chemical stability and lack of nonspecific reactivity to thiol and other functional groups may prove useful for study of compensatory DNA repair pathways dependent on DNA unwinding by WRN or related helicases. In addition to their use as research tools, small-molecular inhibitors of WRN and other helicases may be useful for development of anticancer strategies that rely on synthetic lethality for targeting tumors with preexisting DNA repair deficiencies (32).

Demonstration that very low concentrations of the chemotherapy drug mitomycin C are cytotoxic for Fanconi anemia–mutant cells exposed to the helicase inhibitor may have implications for strategies that target Fanconi anemia DNA repair pathway–deficient tumors. Certain sporadic head and neck, lung, ovarian, cervical, and hematologic cancers are characterized by epigenetic silencing of wild-type Fanconi anemia gene expression (33). It is estimated that 15% of all cancers harbor defects in the Fanconi anemia pathway (34). A significant number of these tumors may become reliant on WRN or other helicases to deal with DNA damage such as strand breaks. WRN may be a suitable target for pharmacologic inhibition to sensitize Fanconi anemia–deficient tumors to chemotherapy drugs such as DNA crosslinkers.

In addition to epigenetic silencing, LOH from an additional mutation in an Fanconi anemia gene in heterozygous carriers may lead to increased cancer risk later in life (33, 35). Fanconi anemia pathway–deficient fibroblasts were found to be highly sensitive to silencing of ATM kinase (33). FANCG- and FANCC–deficient pancreatic tumor lines were sensitive to a pharmacologic inhibitor of ATM, raising the possibility for an

Figure 6. NSC 617145 exposure elicits NHEJ pathway in Fanconi anemia–deficient cells upon cotreatment with mitomycin C. A, FA-D2−/− and FA-D2+/− cells were treated with NSC 617145 (0.125 μmol/L), mitomycin C (9.4 nmol/L), or both compounds for 2 days. Cells were stained with anti-DNA-PKcs-pS2056 antibody or 4′,6-diamidino-2-phenylindole (DAPI). B, percentage of cells with DNA-PKcs-pS2056 foci (≤15 or >15, as indicated) is shown.
anticancer treatment. More recently, Fanconi anemia–deficient cell lines were shown to be hypersensitive to inhibition of CHK1 kinase either by siRNA or a pharmacologic inhibitor of CHK1 kinase activity (36). Unlike the hypersensitivity of Fanconi anemia–deficient cells to CHK1 silencing or kinase inhibition, WRN depletion did not increase sensitivity of FA-D2/C0 cells to mitomycin C. This suggests that NSC 617145 exerted its effect through a dominant-negative mechanism in which NSC 617145–inhibited WRN helicase induced greater damage by blocking compensatory mechanism(s). Inhibition of WRN or a related helicase by a small molecule may provide an alternative strategy for targeting Fanconi anemia–deficient tumors that is unique from approaches such as targeting the CHK1 or ATM kinase response.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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