The Cyclin-Dependent Kinase Inhibitor Flavopiridol Potentiates the Effects of Topoisomerase I Poisons by Suppressing Rad51 Expression in a p53-Dependent Manner

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Abstract

The results of a phase I clinical trial of the topoisomerase I (Topo I) poison CPT-11 followed by the cyclin-dependent kinase inhibitor flavopiridol in patients with advanced solid tumors indicate that patients whose tumors were wild-type, but not mutant, for p53 obtained the most clinical benefit from this combination therapy. We elected to elucidate the mechanistic basis for this effect in isogenic-paired HCT116 colon cancer cells that were either wild-type (+/+) or null (−/−) for p53. With the combination therapy of SN-38 (the active metabolite of CPT-11) followed by flavopiridol, the induction of apoptosis was 5-fold greater in the p53+/+ cells compared with the p53−/− cells. This sequential treatment induced phosphorylation of p53 at Ser^{15}, which interacted with Rad51, a DNA repair protein involved in homologous recombination. Rad51 bound to p53-Ser^{15} within the first 5 hours of combination therapy, and then was transcriptionally suppressed at 24 hours by flavopiridol only in p53+/+ cells. Microarray analysis also revealed suppression of Rad51 in a p53-dependent manner. Depletion of Rad51 by small interfering RNA (siRNA) sensitized both p53+/+ and p53−/− cells to SN-38–induced apoptosis with increase of γH2AX, a marker of DNA damage. Conversely, overexpression of Rad51 rescued p53+/+ cells from SN→F–induced apoptosis. Because flavopiridol inhibits Cdk9, we found that inhibition of Cdk9 by DRB or by siRNA could recapitulate the flavopiridol effects, with suppression of Rad51 and induction of apoptosis only in p53+/+ cells. In conclusion, after DNA damage by Topo I poisons, flavopiridol targets homologous recombination through a p53-dependent down-regulation of Rad51, resulting in enhancement of apoptosis. [Cancer Res 2008;68(7):2312–20]

Introduction

SN-38 (the active metabolite of CPT-11) stabilizes the topoisomerase I (Topo I)–DNA complex, and its collision with the DNA replication fork leads to the generation of permanent strand breaks and G_2 arrest. Flavopiridol, a pan–cyclin-dependent kinase inhibitor, has been reported to induce DNA damage (1) and inhibit tumor cell growth through cell cycle arrest at G_1 or G_2 (2). We have previously reported that flavopiridol at noncytotoxic, nanomolar concentrations significantly enhances the induction of apoptosis by the chemotherapeutic agent mitomycin C and paclitaxel in gastric and breast cancer cell lines (3, 4). Also, the addition of flavopiridol to HCT116 cells enhanced the induction of apoptosis by SN-38 and increased the efficacy of CPT-11 in HCT116-treated xenografts (5). These events were highly sequence dependent such that SN-38 or CPT-11 needed to be administered before flavopiridol both *in vitro* and *in vivo* to induce this effect.

Based on this preclinical data, a phase I clinical trial of CPT-11 followed by flavopiridol was initiated. This study indicated that patients whose tumors were wild-type, but not mutant, for p53 obtained the most clinical benefit from this combination therapy (6). In view of this, we elected to determine the role of p53 in the enhancement of SN-38–induced apoptosis by flavopiridol. After DNA damage, the level of p53 increases and induces a range of responses, including cell cycle arrest, apoptosis, senescence, and DNA repair. This depends on the type and dose of the genotoxic stress. Recent studies have shown that p53 represents a central player in DNA repair by repressing homologous recombination, counteracting error-prone double-strand breaks, and promoting genomic stability (7). Activation of p53 is also mediated by posttranslational modifications by specific kinases, such as the ataxia-telangiectasia mutated (ATM) and the ATM and Rad3 related (8, 9). When DNA replication is blocked, p53 becomes phosphorylated at Ser^{15} and associates with key enzymes of homologous recombination, such as the MRN complex, Rad51 and Rad54 (10, 11). Rad51 is central to homologous recombination–mediated double-strand break repair, by forming nucleoprotein filaments and mediating strand exchange between DNA duplexes (12, 13). Aberrant amounts of Rad51 have been observed in a number of transformed cell types (14) and may induce malignant transformation. Biochemical investigations showed that p53 inhibits Rad51-mediated strand exchange *in vitro* (11, 15, 16). Furthermore, p53 modulates homologous recombination by the transcriptional repression of the *RAD51* gene (17). Therefore, we elected to determine whether the enhancement of SN-38–induced apoptosis was p53 dependent and the role of Rad51 in this process.

Materials and Methods

Cell culture. HCT116 human colon carcinoma cells and its p53-null variant were a gift from Dr. Vogelstein (Johns Hopkins University, Baltimore, MA). Cells were grown in McCoy's 5A medium supplemented with 10% fetal bovine serum. SN-38 (5 mmol/L stock, Pharmacia) was prepared in DMSO. Flavopiridol (4.5 mmol/L) was prepared in water. z-VAD was purchased from MBL International. Cycloheximide and LnII were from Sigma Aldrich.

Apoptosis assays. The measurement of apoptosis by quantitative fluorescence microscopy (QFM) with 4′,6′-diamidino-2-phenylindole (Sigma Chemical Co.) for nuclear chromatin was performed as previously described (3). Apoptosis was also measured using Annexin V–FITC Apoptosis detection Kit II (BD PharMingen) following the manufacturer's instructions. The statistical significance of the experimental results was determined by the two-sided *t* test.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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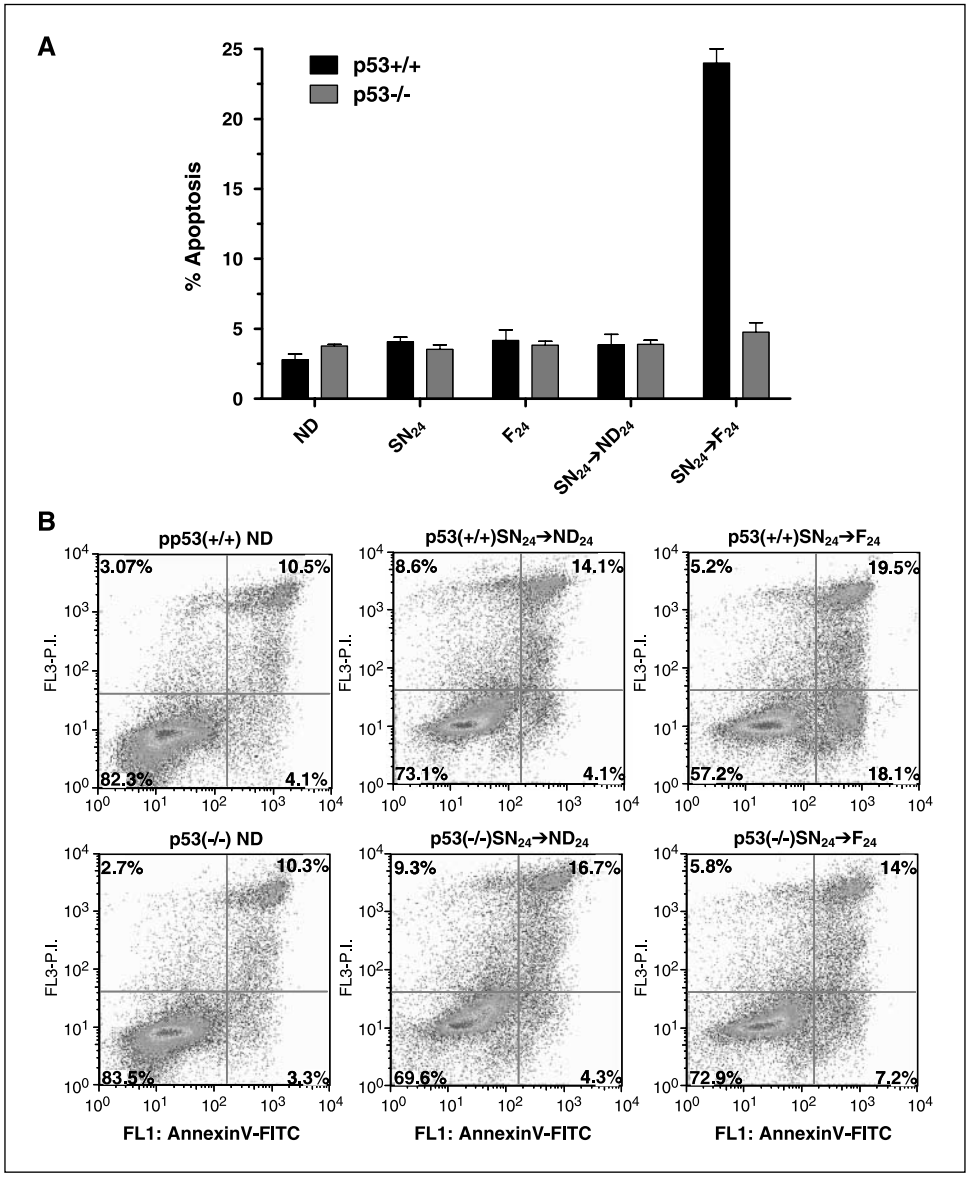

Figure 1. Flavopiridol induces apoptosis in SN-38–treated HCTp53+/+ cells. Cells were treated with SN-38 (20 nmol/L) followed by drug-free medium (*ND*) or flavopiridol (300 nmol/L). Apoptosis was scored by QFM analysis (*A*) or staining with Annexin V (*B*).

Immunoblotting. Cells were lysed in radioimmunoprecipitation assay buffer supplemented with protease inhibitor (Complete Mini, Roche Diagnostics) and 1 mmol/L $NaVO_3$. Equal amounts of protein were loaded on 4% to 12% PAGE gels (Invitrogen). The membranes were probed with antibodies for p53 (Santa Cruz Biotechnology, Inc.), Rad51 (Calbiochem), p53-Ser[15] (Cell Signaling), and α-tubulin (Upstate Biotechnology). Equivalent amounts of proteins from the cleared lysates were incubated with Rad51 antibody for 3 h at 4°C. Then, 30 μL of agarose beads (Upstate Biotechnology) were added for an additional hour. Immunocomplexes were washed in lysis buffer and suspended in 4× SDS sample buffer for Western blot analysis.

Quantitative real-time PCR. RNA was extracted with TriPure Isolation Reagent (Roche Diagnostic). Reverse transcription of 1 μg of RNA was done using the SuperScript III First-Strand Synthesis System (Invitrogen) following the manufacturer's instructions. All quantitative real-time reverse transcription PCR assays were done on the 7300 Real Time PCR System (Applied Biosystems). TaqMan gene expression assays (Applied Biosystems), which include gene-specific probe and primer sets, were used to detect both Rad51 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The relative expression of Rad51 was calculated by normalizing to GAPDH mRNA. Graphs are the means of three separate experiments.

Microarray data analysis. cDNA was synthesized in the presence of oligo(dT)24-T7 from Genset Corp. cRNA was prepared using biotinylated UTP and CTP and was hybridized to HG U113A oligonucleotide arrays (Affymetrix, Inc). Data preprocessing and statistical analysis were carried out in R[3] and Bioconductor.[4] The expression intensities were normalized using the Robust Multiarray Average method (18), which includes (*a*) background adjustment, (*b*) quantile normalization across arrays, and (*c*) probe-level expression measure summarization using median polish on the log 2 scale for each probe set. Differential expression analysis was performed to identify genes that were differentially expressed between SN alone–treated versus SN→F–treated samples in the parental cell line and in the p53-deficient cell line, respectively. An empirical Bayes *t* test was applied to each gene (19). This test is a modified version of the conventional *t* test and is more suitable for data with small sample size. A *P* value cutoff of 0.001 was used to select differentially expressed genes ($P \leq 0.001$), whereas a *P* value cutoff of 0.50 was used to select

[3] http://www.r-project.org
[4] http://www.bioconductor.org

nondifferentially expressed genes ($P \geq 0.50$). Unsupervised hierarchical clustering was done to look for samples and genes with similar gene expression patterns. The samples were clustered using the Euclidean distance as the dissimilarity measure and the Ward function as the joining method; the genes were clustered using the Euclidean distance and the Complete function. The results of the cluster analysis were displayed a heat map.

Plasmid and small interfering RNA transfections. Rad51 full-length cDNA was cloned from HCT116 cells using the Titan One Step reverse transcription-PCR Kit (Roche Applied Science) and inserted in the mammalian expression vector pcDNA3.1 (Invitrogen). The Rad51/P186 mutant (11) was generated using the QuikChange II Site-Directed Mutagenesis Kit (Stratagene). Cdk9 (SMART-Pool) and Rad51 small interfering RNA (siRNA) were obtained from Dharmacon, Inc. The sequence for Rad51 siRNA was GAGCUUGACAAACUACUUC. siRNAs were transfected using OligofectAMINE reagent (Invitrogen).

Results

Flavopiridol enhances SN-38–induced apoptosis in a p53-dependent manner. Human colon carcinoma HCT116 cells with wild-type p53 (p53+/+) and its p53 null isogenic sub–cell line (p53−/−) were treated with 20 nmol/L SN-38 and 300 nmol/L flavopiridol individually and sequentially, as SN-38 for 24 hours (SN_{24}) followed by either flavopiridol ($SN_{24}\rightarrow F_{24}$) or drug-free medium for 24 hours ($SN_{24}\rightarrow ND_{24}$). It has been shown that under these treatment conditions, SN-38 induces DNA damage and a potent G_2 cell cycle arrest in both cell lines at 24 hours, with p53 and p21 up-regulation in the p53+/+ cells (5) and activation of chk1 kinase in the p53−/− cells (20). As determined by QFM (Fig. 1*A*), SN-38 or flavopiridol alone did not induce significant apoptosis. The sequential treatment of p53+/+ cells with $SN_{24}\rightarrow F_{24}$ induced apoptosis in 23.5 ± 2.1% of the cells, which was significantly greater than that observed for $SN_{24}\rightarrow ND_{24}$ (3.8 ± 1.2%). However, the $SN_{24}\rightarrow F_{24}$ treatment induced 4.7 ± 1.1% apoptosis in p53−/− cells. This was further evaluated with Annexin V+ staining (Fig. 1*B*). With this method, 18% to 20% of both p53+/+ and p53−/− cells became positive for Annexin V after SN-38 treatment. With the addition of flavopiridol, Annexin V+ increased to 37.6% in the p53+/+ cells ($P < 0.001$ versus SN-38 alone–treated cells), whereas there was no significant change in the p53−/− cells. Thus, the enhancement of SN-38–induced apoptosis in G_2-arrested cells by flavopiridol seems to be p53 dependent.

In view of this, we investigated the effects of SN-38 and flavopiridol on p53 induction in the p53+/+ cells. As shown in Fig. 2*A*, both conditions up-regulated p53 protein levels over time. In contrast, with SN-38 for 24 hours followed by drug-free medium for up to 24 hours ($SN_{24}\rightarrow ND_{24}$), there was a time-dependent decrease in p53 expression. However, the sequential treatment of SN-38 for 24 hours followed by flavopiridol for 3 to 24 hours ($SN_{24}\rightarrow F_x$) induced a time-dependent increase in total p53, as well as the induction of phosphorylated p53 at Ser^{15}. Under these conditions, Rad51 was induced by SN-38 at 24 hours, which was sustained when the cells were then changed to drug-free medium. In contrast, Rad51 was down-regulated by flavopiridol, both as a single agent and in sequential treatment with SN-38 ($SN_{24}\rightarrow F_x$).

Phosphorylated p53 at Ser^{15} has been found to interact with Rad51 and inhibit its activity during DNA repair (10). Interestingly, coimmunoprecipitation experiments (Fig. 2*B, right*) showed that p53 bound to Rad51 after 5 hours of the sequential $SN\rightarrow F_5$ treatment, but not with SN-38 alone for 24 hours ($SN\rightarrow F_0$). This interaction was sustained for the next 16 hours, until Rad51 was down-regulated at 24 hours by flavopiridol (Fig. 2*B*).

Flavopiridol down-regulates Rad51 in a p53-dependent manner. Thus, it would seem that with this sequential therapy at early time points, Rad51 binds to p53-Ser^{15}; however Rad51 expression is suppressed over time. Rad51 is an important regulator of homologous recombination and is reportedly inhibited by p53, not only through physical interaction but also by transcriptional repression (17). Thus, we tested whether

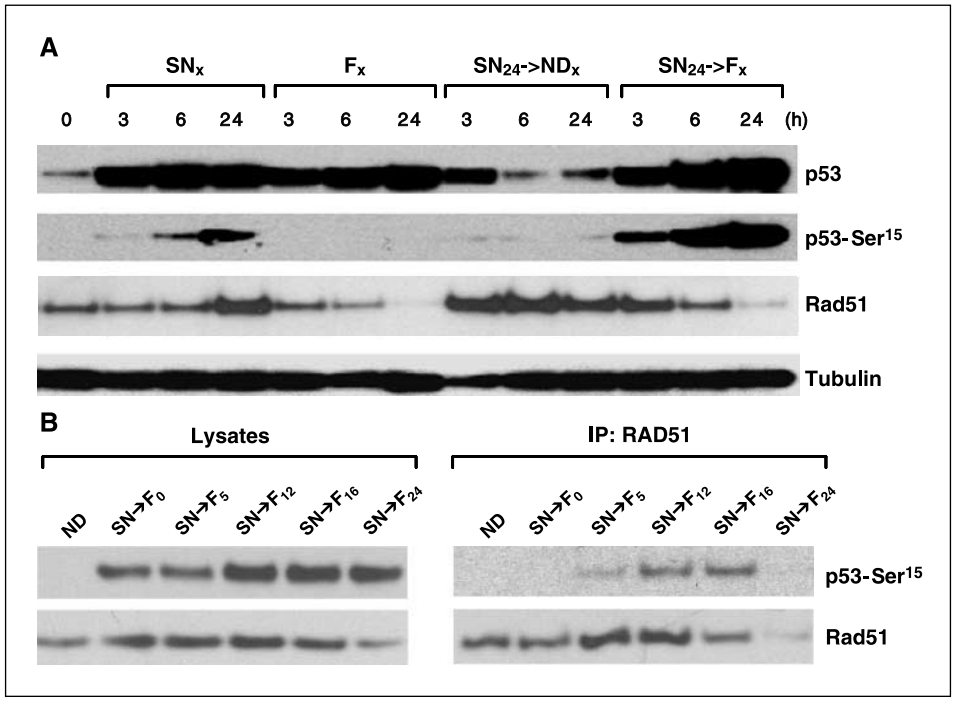

Figure 2. After DNA damage by SN-38, flavopiridol induces p53 phosphorylation on Ser^{15} and down-regulates Rad51. *A,* Western blotting of cells treated with SN-38 and flavopiridol at various time points or SN-38 for 24 h (SN_{24}) followed by drug-free medium or flavopiridol (*F*) at various time points. Tubulin is shown to confirm equal protein loading. *B,* Rad51 binds to p53-Ser^{15}. HCTp53+/+ cells were treated with drug-free medium or SN-38 for 24 h followed by flavopiridol over the time. Equal amounts of the cell lysates were immunoprecipitated with an anti-Rad51 antibody and then blotted with anti–p53-Ser^{15} or Rad51 antibodies (*right*). As a control, the total lysates were blotted with the same antibodies (*left*).

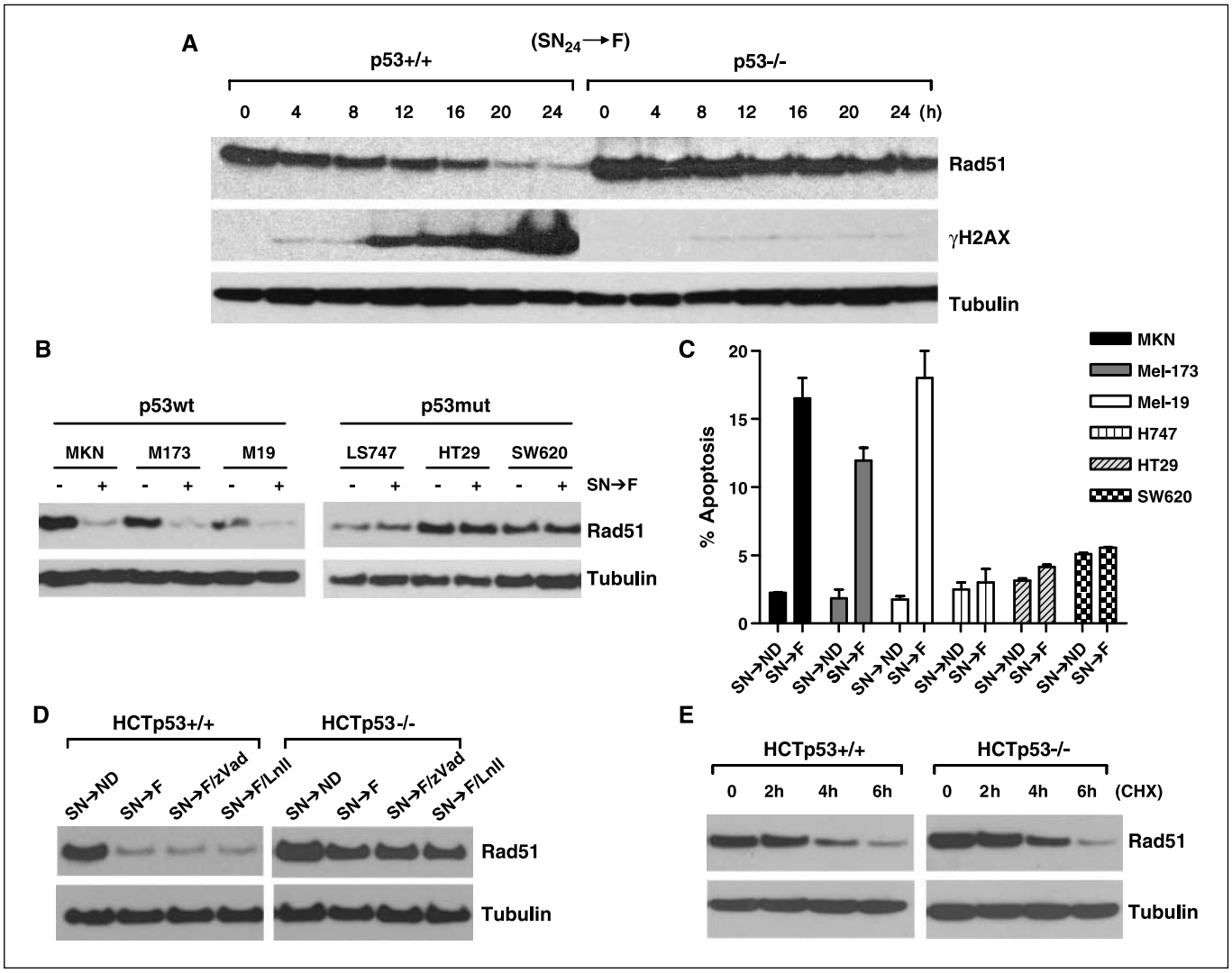

Figure 3. Flavopiridol induces down-regulation of Rad51 in a p53-dependent manner. *A,* HCTp53+/+ and p53−/− cells were treated with SN-38 for 24 h, then released in drug-free medium or flavopiridol for the indicated times. Lysates were analyzed by immunoblotting for Rad51 and γH2AX expression. *B,* a series of cell lines with wild-type or mutant p53 was treated with SN→ND (−) and SN→F (+) for 24 h and analyzed for Rad51 expression. Tubulin is shown to confirm equal protein loading. *C,* identical conditions were used to evaluate these same cell lines for apoptosis by QFM. *D,* cells were treated SN→F in the presence of 10 μmol/L zVAD or 5 μmol/L LnII and analyzed by Western blotting. *E,* HCTp53+/+ and p53−/− cells were treated SN→F in the presence of cycloheximide for the indicated times. *CHX,* cycloheximide.

flavopiridol could differentially regulate Rad51 depending on the p53 status of the cells. HCTp53+/+ and p53−/− cells were treated with SN-38 for 24 hours followed by flavopiridol for the indicated times. Rad51 declined in p53+/+ cells, with almost complete down-regulation after 20 hours of flavopiridol treatment (Fig. 3*A*). In contrast, Rad51 continued to be expressed over time in p53−/− cells. In addition, γ-H2AX showed a time-dependent increase only in the p53+/+ cells, consistent with an increase in double-strand DNA breaks.

To extend these findings for the p53 dependency of Rad51 suppression, we tested the effect of SN-38 and flavopiridol on a panel of cell lines that differ in their p53 status. As shown in Fig. 3*B*, the suppression of Rad51 with the sequential therapy of SN→F was observed only in cell lines that are wild-type for p53, including gastric cancer (MKN-74) and melanomas (M173 and M19). Still, no suppression of Rad51 was observed in three colon cancer cell lines (H747, HT29, and SW620) that are mutant for p53. This is in contrast to the HCTp53−/− cells, which exhibited a decrease in Rad51 expression at later time points, suggesting slight differences in cell-specific responses to flavopiridol among p53 mutant cell lines. The suppression of Rad51 expression in the p53 wild-type cell lines again correlated with the enhancement of SN-38–induced apoptosis by QFM (Fig. 3*C*). In contrast, there was no increase in apoptosis with SN→F in the p53 mutant cell lines. In control experiments, to exclude proteasome- and caspase-mediated effects on Rad51 down-regulation, we used the pan-caspase inhibitor z-VAD or the proteasome inhibitor LnII together with the SN→F treatments. Under these conditions, the time-dependent suppression of Rad51 by SN→F in the p53+/+ cells could not be restored by the addition of either compounds (Fig. 3*D*). Furthermore, Rad51 protein stability was assessed in both cell lines by adding cycloheximide to the SN→F treatments, and we found no difference in protein decay by 6 hours (Fig. 3*E*).

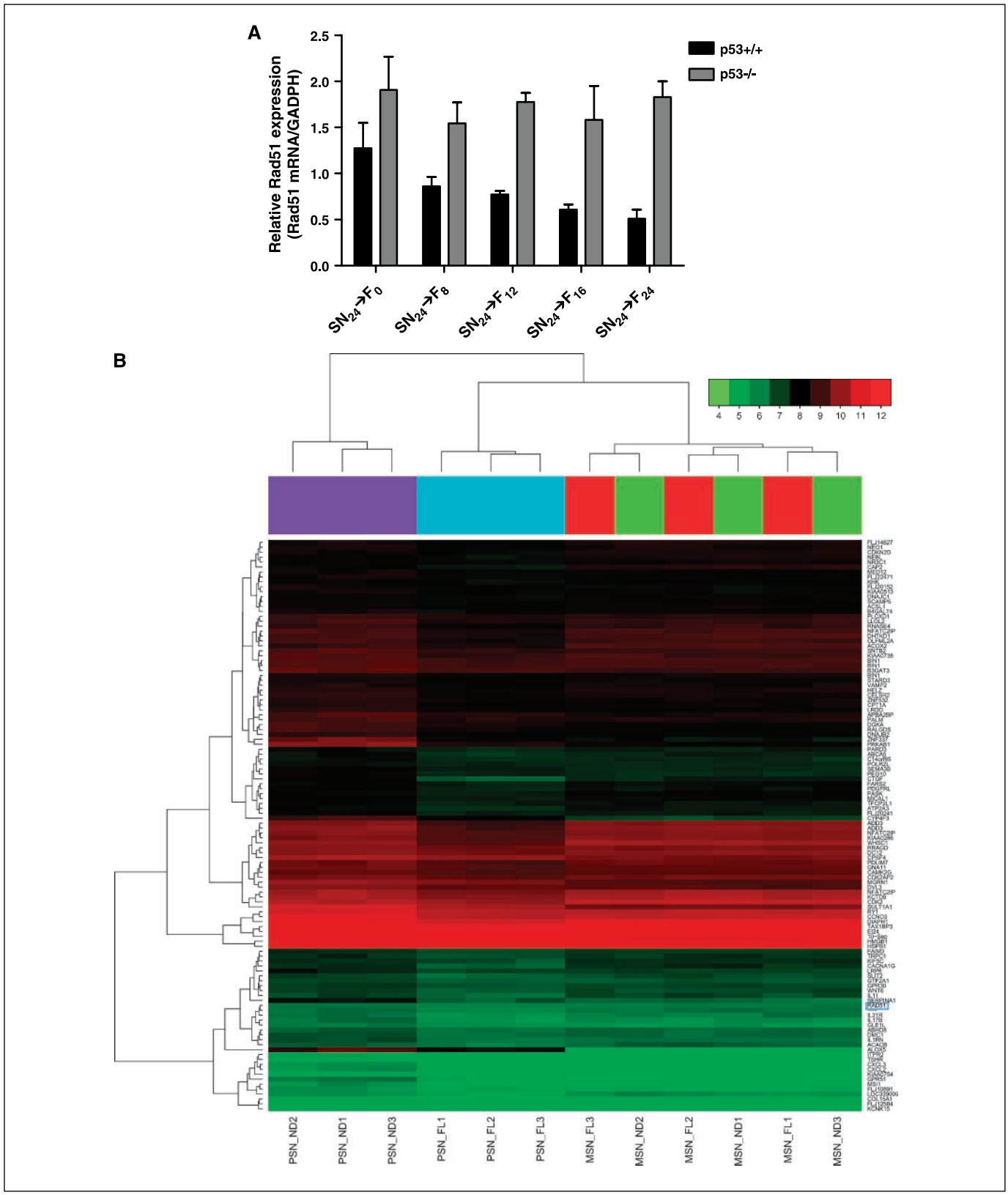

Figure 4. *A,* quantitative RT-PCR analysis of Rad51 expression in HCTp53+/+ and p53−/− cells treated with 20 nmol/L SN-38 for 24 h followed by 300 nmol/L flavopiridol for various time intervals. *Columns,* mean of relative Rad51 expression after normalizing to the levels of *GADPH* gene (relative to untreated controls = 1). Means were obtained from three independent experiments done in triplicate. *B,* genes differentially suppressed by flavopiridol, comparing the parental cell line (*P*) with the p53-deficient cell line (*M*), treated with SN→ND and SN→F. A *P* value cutoff of 0.001 was used to select differentially expressed genes in the parental cell line; a *P* value cutoff of 0.50 was used to select nondifferentially expressed genes in the p53-deficient cell line.

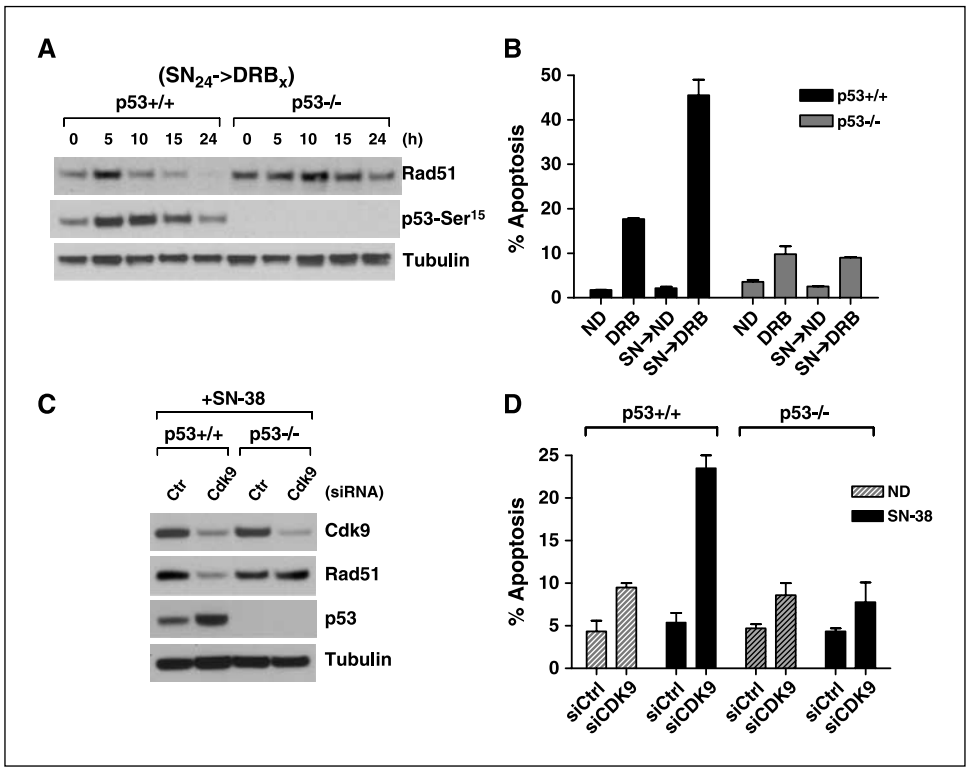

Figure 5. *A,* inhibition of Cdk9 partially down-regulates Rad51 and induces p53-dependent apoptosis. Western blot for Rad51 expression and p53-Ser[15] in HCT p53+/+ and p53−/− cells treated with 20nmol/L SN-38 followed by drug-free medium or by 50 mmol/L DRB for the indicated times. *B,* QFM analysis for apoptosis of cells subjected to same treatments. *C,* cells were transfected with Cdk9 siRNA, treated 24 h later with SN-38, and analyzed for protein expression and apoptosis (*D*). *Columns,* mean of three independent experiments performed in duplicates.

Next, we compared Rad51 mRNA levels by real-time PCR in both cell lines. The addition of flavopiridol to the SN-38–treated cells induced significant reductions in Rad51 mRNA in the p53+/+ cells over time (Fig. 4*A*). In contrast, Rad51 mRNA levels did not decrease significantly with flavopiridol in the p53−/− cells. To further investigate the effect of flavopiridol on the expression of p53-dependent genes, we performed microarray analysis, using Affymetrix U133A GeneChip arrays, to identify genes that were differentially expressed between SN-38 alone versus SN→F–treated samples in the p53+/+ cells, but not in the p53−/− cell line. There are 116 genes that are down-regulated by flavopiridol in the HCTp53+/+ cell line but unaffected in the HCTp53−/− cells, which we will call "differentially suppressed genes" (Supplementary Data). Among the 116 genes, an unsupervised hierarchical clustering was performed to look for samples and genes with similar expression patterns (Fig. 4*B*). Some of these differentially expressed genes encode proteins critical for cell cycle (*Cdk2, cyclin D3, p19*), regulation of transcription (*GTF2A1, NR3C1, POLR2L, MED12, ZNF337, DNAJC1*), and regulation of apoptosis (*FAIM3*). We also found significant suppression of two genes involved in DNA repair, one of them being *Rad51*. The other is *HMGB1*, a member of the large family of HMG-box proteins, which has been associated with mammalian excision base repair in the setting of oxidative stress (21). Taken together, these results suggest that Rad51 is down-regulated by flavopiridol in a p53-dependent manner. Furthermore, a survey of genes indicates that *Rad51* is the only gene suppressed in a p53-dependent manner that has been directly linked to the process of homologous recombination.

The suppression of Rad51 is, in part, mediated by inhibition of Cdk9. Flavopiridol is a potent inhibitor of Cdk9, which phosphorylates the COOH-terminal domain of RNA polymerase II and promotes initiation and elongation of mRNA transcripts (22). To address the possibility that Cdk9 inhibition by flavopiridol may directly suppress the expression of Rad51, we used a specific Cdk9 inhibitor, DRB, and a specific Cdk9 siRNA in HCTp53+/+ and HCTp53−/− cells. Both DRB (Fig. 5*A*) and Cdk9 depletion by siRNA (Fig. 5*C*) mimicked flavopiridol effects in SN-38–treated p53+/+ cells, with down-regulation of Rad51, induction of p53, and increase in apoptosis (Fig. 5*A–D*). In p53−/− cells, however, Rad51 did not change after Cdk9 down-regulation by siRNA (Fig. 5*C*), slightly decreased only at later time points with SN→DRB treatment (Fig. 5*A*), and demonstrated no significant increase in apoptosis (Fig. 5*B* and *D*). These results suggest that down-regulation of Rad51 by flavopiridol is, at least in part, due to the inhibition of Cdk9, and that the induction of apoptosis under these treatment conditions still requires the presence of p53.

Depletion of Rad51 sensitizes HCT116 cells to single-agent SN-38. Next, we tested whether the depletion of Rad51 would recapitulate the effect of flavopiridol, in the presence or absence of p53. Both cell lines were transfected with a specific Rad51 or control siRNA and then treated with SN-38. As shown in Fig. 6*A*, Rad51 was completely down-regulated in both p53+/+ and p53−/− cell lines after siRNA transfection. There was evidence of induction of γH2AX (Fig. 6*A*) and low levels of spontaneous apoptosis in both cell lines (Fig. 6*B*) when compared with siRNA controls. Treatment with SN-38 for 24 hours (SN_{24}) induced 23.7 ± 1.7% apoptosis in p53+/+ cells and 19.2 ± 0.3% in p53−/− cells (Fig. 6*B*). These results suggest that Rad51 plays a role in the protection from apoptosis to single-agent SN-38 treatment in both cell lines.

Overexpression of Rad51 results in resistance to SN→F according to p53 status. The role of Rad51 in SN→F–induced apoptosis was further investigated by generating Rad51-overexpressing cells. In these experiments, we also used the mutant

Rad51/186P (11), which lacks the ability to bind p53. The constructs were transfected in p53+/+ cells 24 hours before SN→F treatment. Rad51 protein levels are shown in Fig. 6*C*. Overexpression of Rad51 and Rad51/P186 decreased the percentage induction of apoptosis by SN→F from 21 ± 1% of vector transfectants to 11 ± 1% and 8 ± 1%, respectively (Fig. 6*D*). This also corresponded to a decrease in γH2AX expression, demonstrating that with SN→F, Rad51 overexpression decreased DNA damage, especially when it could not bind to p53. Taken together, these results indicate that Rad51 plays a central role in the resistance to SN-38 and that flavopiridol induces a p53-dependent suppression of Rad51 that results in induction of apoptosis.

Discussion

Homologous recombination mediates the error-free repair of DNA, mostly during the late S-G_2 phases of the cell cycle (23, 24). In the presence of high doses of DNA-damaging agents or defects in the DNA repair machinery, the repair is unsuccessful and this may lead to permanent cell cycle arrest or apoptosis. Our studies indicate that flavopiridol converted Topo I–induced cell cycle arrest into apoptosis through a p53-dependent transcriptional suppression of Rad51. This seems to involve two steps. First, with sequential SN→F treatment, there is early binding of p53-Ser^{15} to Rad51, which seems to be sufficient to inhibit the process of Rad51-mediated DNA repair that occurs after SN-38–induced DNA damage. In fact, mutation of Rad51 at its binding domain to p53 significantly prevented the induction of apoptosis with the sequential therapy. This is then followed by a second event of down-regulation of Rad51 at the mRNA level.

Our studies indicate that this effect is not due to caspase- or proteasome-mediated degradation of Rad51 or due to differences in protein stability. This transcriptional suppression of Rad51 with sequential SN→F and the resulting induction of apoptosis are absolutely dependent on the presence of wild-type p53. This p53-dependent effect on Rad51 suppression was further confirmed using an unsupervised hierarchical clustering of gene expression profiles. Among 116 genes suppressed by flavopiridol on SN-38–treated HCTp53+/+ cells, only two genes involved in DNA repair were identified, and one of them was *Rad51*. Although these results do not exclude the effect of the transcriptional suppression of these other genes in this combination therapy, it indicates that when flavopiridol is combined with a topoisomerase poison the suppression of Rad51 does play a significant role in the enhancement chemotherapy-induced apoptosis.

Flavopiridol has been implicated in the transcriptional repression of numerous genes (25), including the antiapoptotic genes *Mcl-1* (26), the *X-linked inhibitor of apoptosis protein* (*XIAP*), and *c-Kit* (27). This effect is mediated by inhibition of the Cdk9/pTEFb complex, which regulates RNA polymerase II (21). These effects have not being reported as p53 dependent. In fact, our microarray analysis of genes suppressed in a p53-dependent manner with SN→F did not indicate differential suppression of Mcl-1 or any of the Bcl-2 family members. Using siRNA against Cdk9 and DRB to target Cdk9 with SN-38, we show suppression of Rad51 in the p53+/+ cells. This would suggest that suppression of Rad51 by Cdk9 inhibition is a p53-dependent process and that flavopiridol represents a novel therapeutic approach in the targeting of this gene.

In the p53+/+ cells, both SN-38 and flavopiridol are shown to up-regulate p53. However, it is only after SN→F treatment that we observe sustained p53 phosphorylation. It has been shown that p53 can reduce the activity of a variety of promoters (28), including those of c-fos (29) and Rb (30). Posttranslational modifications of

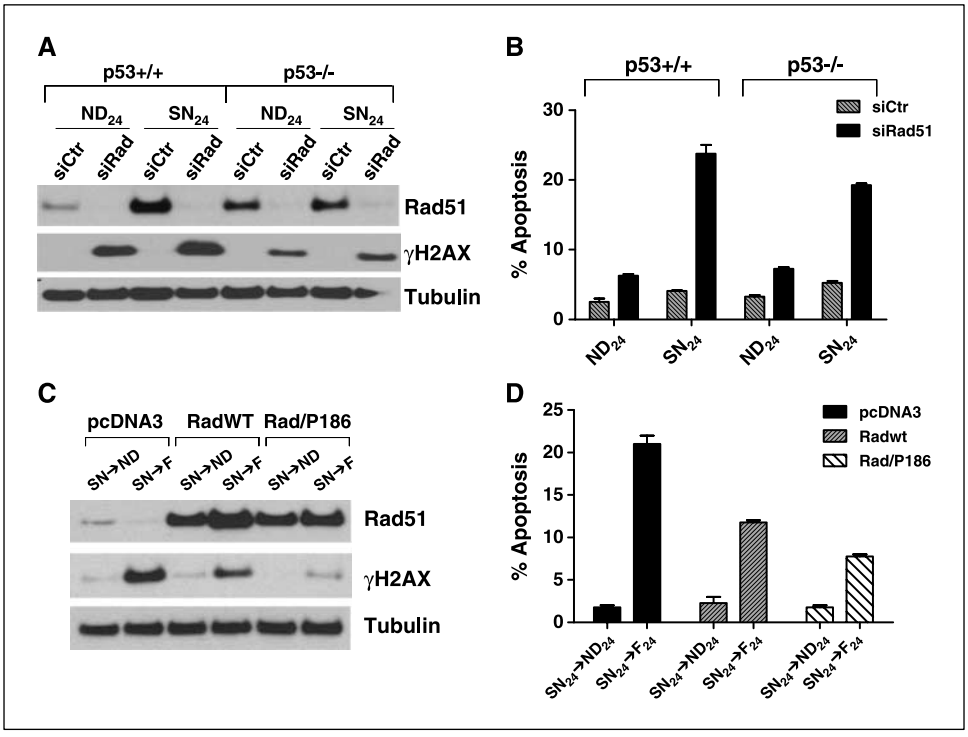

Figure 6. Rad51 depletion by siRNA sensitizes cells to SN-38. *A,* HCTp53+/+ and p53−/− cells were transfected with control and Rad51 siRNA. Western blot analysis shows Rad51 knockdown in both SN-38–treated or untreated cells. γH2AX was used to detect DNA damage, and tubulin was the loading control. *B,* apoptosis was determined by QFM after the indicated treatments. Experiments were carried out thrice in duplicates. Overexpression of Rad51 protects from SN→F–induced apoptosis. *C,* HCTp53+/+ cells were transfected with Rad51, the mutant Rad/P186, or the empty vector pcDNA3. Cell lysates were subject to immunoblotting using Rad51, γH2AX, and tubulin antibodies. *D,* apoptosis assay of transfected cells after treatments. *Columns,* mean of three independent experiments.

p53, such as phosphorylation and acetylation, are involved in the modulation of p53 transcriptional activities (31, 32). This increases the possibility that p53 in its phosphorylated state may be playing a role in the transcriptional suppression of Rad51 under these treatment conditions.

Recently, inhibition of cyclin-dependent kinases has been implicated in the reduction of DNA repair mechanisms. For example, the pan–cyclin-dependent kinase inhibitor roscovitine sensitized tumor cell lines to doxorubicin by increasing the amount of double-strand DNA breaks and reducing the efficiency of homologous recombination repair (33). This effect seemed to be predominant in the G_2-M phase of the cell cycle, where homologous recombination was preferentially active. Although DNA-PK activity was suppressed by roscovitine, the effect of roscovitine on Rad51 was not explored.

Rad51 activity is essential during homologous recombination–mediated double-strand break repair, either during replication (34) or following exposure to DNA-damaging agents. Its central role in homologous recombination is supported by studies with Rad51 knockout mice, which showed early embryonic lethality (35). Furthermore, it has been reported that down-regulation of Rad51 enhanced the anticancer activities of cisplatin (36) and increased the radiosensitivity of tumor cells (37). Conversely, a number of studies showed that high levels of Rad51 caused resistance to a variety of chemotherapeutic agents like doxorubicin (38) and 5-fluorouracil (39). We also found that overexpression of Rad51 significantly protected p53+/+ cells from SN→F–induced apoptosis.

For flavopiridol, the targeting of Rad51 seems to be critical for the increase in DNA damage and the enhancement of SN-38–induced apoptosis. This observation and a mechanistic understanding of this process should have significant implications for the development of this agent in solid tumors. Currently, flavopiridol has been shown to have single activity in chronic lymphocytic leukemia, which is believed to be mediated by the flavopiridol targeting of Cdk9 and the suppression of Mcl-1 and bcl-2 family members (40). However, the experience with single-agent flavopiridol in the treatment of solid tumors has been generally disappointing (41, 42). In contrast, clinical activity of flavopiridol in combination with chemotherapy has been observed (6, 43, 44). The ultimate success of this agent will depend on the identification of subpopulation of patients who will most benefit from the combination with chemotherapy. A clinical trial to test this hypothesis with sequential CPT-11 and flavopiridol in patients with gastroesophageal cancer who are wild-type p53 is now being planned, and suppression of Rad51 in serial tumor biopsies will be examined. Therefore, based on these laboratory studies, it is our hope that the targeting of Rad51 with Topo I inhibitors followed by flavopiridol will represent a new approach for the treatment of solid tumors that are wild-type p53.

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References

1. Bible KC, Bible RH, Jr., Kottke TJ, et al. Flavopiridol binds to duplex DNA. Cancer Res 2000;60:2419–28.
2. Kaur G, Stetler-Stevenson M, Sebers S, et al. Growth inhibition with reversible cell cycle arrest of carcinoma cells by flavone L86–8275. J Natl Cancer Inst 1992;84:1736–40.
3. Schwartz GK, Farsi K, Maslak P, Kelsen DP, Spriggs D. Potentiation of apoptosis by flavopiridol in mitomycin-C–treated gastric and breast cancer cells. Clin Cancer Res 1997;3:1467–72.
4. Motwani M, Delohery TM, Schwartz GK. Sequential dependent enhancement of caspase activation and apoptosis by flavopiridol on paclitaxel-treated human gastric and breast cancer cells. Clin Cancer Res 1999;5:1876–83.
5. Motwani M, Jung C, Sirotnak FM, et al. Augmentation of apoptosis and tumor regression by flavopiridol in the presence of CPT-11 in Hct116 colon cancer monolayers and xenografts. Clin Cancer Res 2001;7:4209–19.
6. Shah MA, Kortmansky J, Motwani M, et al. A phase I clinical trial of the sequential combination of irinotecan followed by flavopiridol. Clin Cancer Res 2005;11:3836–45.
7. Gatz SA, Wiesmuller L. p53 in recombination and repair. Cell Death Differ 2006;13:1003–16.
8. Canman CE, Lim DS, Cimprich KA, et al. Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science 1998;281:1677–9.
9. Tibbetts RS, Brumbaugh KM, Williams JM, et al. A role for ATR in the DNA damage-induced phosphorylation of p53. Genes Dev 1999;13:152–7.
10. Restle A, Janz C, Wiesmuller L. Differences in the association of p53 phosphorylated on serine 15 and key enzymes of homologous recombination. Oncogene 2005;24:4380–7.
11. Linke SP, Sengupta S, Khabie N, et al. p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. Cancer Res 2003;63:2596–605.
12. Baumann P, Benson FE, West SC. Human Rad51 protein promotes ATP-dependent homologous pairing and strand transfer reactions *in vitro*. Cell 1996;87:757–66.
13. Gupta RC, Bazemore LR, Golub EI, Radding CM. Activities of human recombination protein Rad51. Proc Natl Acad Sci U S A 1997;94:463–8.
14. Raderschall E, Stout K, Freier S, Suckow V, Schweiger S, Haaf T. Elevated levels of Rad51 recombination protein in tumor cells. Cancer Res 2002;62:219–25.
15. Yoon D, Wang Y, Stapleford K, Wiesmuller L, Chen J. P53 inhibits strand exchange and replication fork regression promoted by human Rad51. J Mol Biol 2004;336:639–54.
16. Sengupta S, Linke SP, Pedeux R, et al. BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination. EMBO J 2003;22:1210–22.
17. Arias-Lopez C, Lazaro-Trueba I, Kerr P, et al. p53 modulates homologous recombination by transcriptional regulation of the RAD51 gene. EMBO Rep 2006;7:219–24.
18. Irizarry RA, Hobbs B, Collin F, et al. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003;4:249–64.
19. Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. Stat Appl Genet Mol Biol 2004;3:Article3.
20. Tse AN, Schwartz GK. Potentiation of cytotoxicity of topoisomerase I poison by concurrent and sequential treatment with the checkpoint inhibitor UCN-01 involves disparate mechanisms resulting in either p53-independent clonogenic suppression or p53-dependent mitotic catastrophe. Cancer Res 2004;64:6635–44.
21. Prasad R, Liu Y, Deterding LJ, et al. HMGB1 is a cofactor in mammalian base excision repair. Mol Cell 2007;27:829–41.
22. Chao SH, Price DH. Flavopiridol inactivates P-TEFb and blocks most RNA polymerase II transcription *in vivo*. J Biol Chem 2001;276:31793–9.
23. Cahill D, Connor B, Carney JP. Mechanisms of eukaryotic DNA double strand break repair. Front Biosci 2006;11:1958–76.
24. Durant ST, Nickoloff JA. Good timing in the cell cycle for precise DNA repair by BRCA1. Cell Cycle 2005;4:1216–22.
25. Lu X, Burgan WE, Cerra MA, et al. Transcriptional signature of flavopiridol-induced tumor cell death. Mol Cancer Ther 2004;3:861–72.
26. Gojo I, Zhang B, Fenton RG. The cyclin-dependent kinase inhibitor flavopiridol induces apoptosis in multiple myeloma cells through transcriptional repression and down-regulation of Mcl-1. Clin Cancer Res 2002;8:3527–38.
27. Sambol EB, Ambrosini G, Geha RC, et al. Flavopiridol targets c-KIT transcription and induces apoptosis in gastrointestinal stromal tumor cells. Cancer Res 2006;66:5858–66.
28. Ginsberg D, Mechta F, Yaniv M, Oren M. Wild-type p53 can down-modulate the activity of various promoters. Proc Natl Acad Sci U S A 1991;88:9979–83.
29. Kley N, Chung RY, Fay S, Loeffler JP, Seizinger BR. Repression of the basal c-fos promoter by wild-type p53. Nucleic Acids Res 1992;20:4083–7.
30. Shiio Y, Yamamoto T, Yamaguchi N. Negative regulation of Rb expression by the p53 gene product. Proc Natl Acad Sci U S A 1992;89:5206–10.
31. Li DW, Liu JP, Schmid PC, et al. Protein serine/threonine phosphatase-1 dephosphorylates p53 at Ser-15 and Ser-37 to modulate its transcriptional and apoptotic activities. Oncogene 2006;25:3006–22.
32. Roy S, Tenniswood M. Site specific acetylation of p53 directs selective transcription complex assembly. J Biol Chem 2006;282:4765–71.

33. Crescenzi E, Palumbo G, Brady HJ. Roscovitine modulates DNA repair and senescence: implications for combination chemotherapy. Clin Cancer Res 2005; 11:8158–71.
34. Lundin C, Schultz N, Arnaudeau C, Mohindra A, Hansen LT, Helleday T. RAD51 is involved in repair of damage associated with DNA replication in mammalian cells. J Mol Biol 2003;328:521–35.
35. Sonoda E, Sasaki MS, Buerstedde JM, et al. Rad51-deficient vertebrate cells accumulate chromosomal breaks prior to cell death. EMBO J 1998;17:598–608.
36. Ito M, Yamamoto S, Nimura K, Hiraoka K, Tamai K, Kaneda Y. Rad51 siRNA delivered by HVJ envelope vector enhances the anti-cancer effect of cisplatin. J Gene Med 2005;7:1044–52.
37. Collis SJ, Tighe A, Scott SD, Roberts SA, Hendry JH, Margison GP. Ribozyme minigene-mediated RAD51 down-regulation increases radiosensitivity of human prostate cancer cells. Nucleic Acids Res 2001;29:1534–8.
38. Abe H, Wada M, Kohno K, Kuwano M. Altered drug sensitivities to anticancer agents in radiation-sensitive DNA repair deficient yeast mutants. Anticancer Res 1994;14:1807–10.
39. Henning W, Sturzbecher HW. Homologous recombination and cell cycle checkpoints: Rad51 in tumour progression and therapy resistance. Toxicology 2003;193: 91–109.
40. Byrd JC, Lin TS, Dalton JT, et al. Flavopiridol administered using a pharmacologically derived schedule is associated with marked clinical efficacy in refractory, genetically high-risk chronic lymphocytic leukemia. Blood 2007;109:399–404.
41. Schwartz GK, Ilson D, Saltz L, et al. Phase II study of the cyclin-dependent kinase inhibitor flavopiridol administered to patients with advanced gastric carcinoma. J Clin Oncol 2001;19:1985–92.
42. Shapiro GI, Supko JG, Patterson A, et al. A phase II trial of the cyclin-dependent kinase inhibitor flavopiridol in patients with previously untreated stage IV non-small cell lung cancer. Clin Cancer Res 2001;7:1590–9.
43. Schwartz GK, O'Reilly E, Ilson D, et al. Phase I study of the cyclin-dependent kinase inhibitor flavopiridol in combination with paclitaxel in patients with advanced solid tumors. J Clin Oncol 2002;20:2157–70.
44. Fornier MN, Rathkopf D, Shah M, et al. Phase I dose-finding study of weekly docetaxel followed by flavopiridol for patients with advanced solid tumors. Clin Cancer Res 2007;13:5841–6.