

DNA Repair Functions That Control Sensitivity to Topoisomerase-Targeting Drugs

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DNA topoisomerases play critical roles in a wide range of cellular processes by altering DNA topology to facilitate replication, transcription, and chromosome segregation. Topoisomerases alter DNA topology by introducing transient DNA strand breaks that involve a covalent protein DNA intermediate. Many agents have been found to prevent the religation of DNA strand breaks induced by the enzymes, thereby converting the enzymes into DNA-damaging agents. Repair of the DNA damage induced by topoisomerases is significant in understanding drug resistance arising following treatment with topoisomerase-targeting drugs. We have used the fission yeast *Schizosaccharomyces pombe* to identify DNA repair pathways that are important for cell survival following drug treatment. *S. pombe* strains carrying mutations in genes required for homologous recombination such as *rad22A* or *rad32* (homologues of *RAD52* and *MRE11*) are hypersensitive to drugs targeting either topoisomerase I or topoisomerase II. In contrast to results observed with *Saccharomyces cerevisiae*, *S. pombe* strains defective in nucleotide excision repair are also hypersensitive to topoisomerase-targeting agents. The loss of DNA replication or DNA damage checkpoints also sensitizes cells to both topoisomerase I and topoisomerase II inhibitors. Finally, repair genes (such as the *S. pombe rad8⁺* gene) with no obvious homologs in other systems also play important roles in causing sensitivity to topoisomerase drugs. Since the pattern of sensitivity is distinct from that seen with other systems (such as the *S. cerevisiae* system), our results highlight the usefulness of *S. pombe* in understanding how cells deal with the unique DNA damage induced by topoisomerases.

Topoisomerases are cellular enzymes that modify the topological state of DNA and participate in metabolic processes such as replication, recombination, transcription, and chromosome segregation (26, 44). These enzymes work by breaking the DNA backbone, carrying out strand passage through the broken DNA, and resealing the break. DNA cleavage by topoisomerases occurs by the introduction of DNA single- or double-strand breaks by a transesterification reaction, in which a tyrosine residue forms a catalytic intermediate that includes a covalent bond between the enzyme and DNA (44).

In addition to their critical biological functions, topoisomerases are the targets of a wide range of antibacterial and antitumor agents. For example, eukaryotic topoisomerase I is the target of camptothecin analogs and agents such as fluoroquinolones and anthracyclines target topoisomerase II (8, 11, 19, 28). These antibacterial and antitumor drugs interfere with the catalytic cycle of topoisomerases by elevating the levels of the covalent complex formed between the enzymes and the cleaved DNA. Agents such as etoposide and amsacrine elevate the levels of covalent complexes by inhibiting the topoisomerase-mediated religation of the cleaved DNA (37). The enzyme:DNA covalent complex, although reversible, can be converted into irreversible DNA damage by various metabolic processes. For example, topoisomerase I covalent complexes can be converted into DNA double-strand breaks by collision with a replication fork (9, 15, 32, 46). In addition to the results seen with the small molecules described above, other processes can also trap topo-

isomerase:DNA covalent complexes. Recent work has suggested that both topoisomerase I and topoisomerase II can be trapped on DNA by various types of DNA damage (18, 25, 30, 36).

The wide range of agents that are able to trap topoisomerase:DNA covalent complexes suggests that cells likely possess multiple mechanisms for repairing or tolerating this unique type of DNA damage. Studies in budding yeast have shown that double-strand break repair pathways are involved in the repair of topoisomerase-mediated DNA damage (12, 24) consistent with the generation of double-strand breaks in DNA following collision between DNA tracking proteins and topoisomerase covalent complexes. It is less clear how the covalent protein:DNA adduct is processed. Nash and colleagues recently identified an enzyme, tyrosyl-DNA phosphodiesterase (TDP), from yeast that is able to remove proteins covalently bound to the 3' but not the 5' end of DNA (35, 45). This enzyme has been subsequently found in a wide range of eukaryotes (16). However, yeast cells lacking *TDPI* have only a slight increase in camptothecin sensitivity, indicating that other pathways likely play roles in repairing protein DNA adducts (35, 39). Since Tdp1p is inactive against protein covalently bound to the 5' end of DNA, this protein would not be expected to play a role in the repair of topoisomerase II covalent complexes.

The budding yeast *Saccharomyces cerevisiae* has been a genetically tractable model of choice for studying the action of topoisomerase-targeting agents. To extend the range of genetically tractable models of anticancer drug action, we have turned to the fission yeast *Schizosaccharomyces pombe*. *S. pombe* is a well-established system for studying various aspects of DNA repair and cellular responses to DNA damage. Since many of the genes that sensitize *S. pombe* to DNA-damaging agents were independently described and named, the naming of genes

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TABLE 1. *S. pombe* genes involved in DNA repair or DNA damage tolerance and their homologs in *S. cerevisiae*

<i>S. pombe</i> gene	<i>S. cerevisiae</i> homolog	Pathway or biochemical activity
<i>rad13</i> ^{Sp}	<i>RAD2</i>	Excision repair, SS annealing
<i>rad2</i> ^{Sp}	<i>RAD27</i>	Flap endonuclease
<i>uve1</i> ^{Sp}	Absent	UV endonuclease
<i>rad22A</i> ^{Sp}	<i>RAD52</i>	Homologous recombination
<i>Rad32</i> ^{Sp}	<i>MRE11</i>	Homologous recombination
<i>rhp51</i> ^{Sp}	<i>RAD51</i>	Homologous recombination
<i>rhp54</i> ^{Sp}	<i>RAD54</i>	Homologous recombination
<i>rad1</i> ^{Sp}	<i>RAD17</i>	DNA damage checkpoint
<i>rad9</i> ^{Sp}	<i>DDC1</i>	DNA damage checkpoint
<i>rad8</i> ^{Sp}	Unknown	Unknown

between *S. pombe* and *S. cerevisiae* are not consistent. Table 1 provides a concordance of important repair genes from *S. pombe* and *S. cerevisiae*. This organism has been used previously to establish topoisomerase I as the sole target of camptothecins (12) and to examine the role of *Chk1* in sensitivity to topoisomerase I targeting agents (43), but repair-deficient mutants have not been systematically examined for sensitivity to either topoisomerase I- or topoisomerase II-targeting agents. Additionally, potent specific drugs targeting topoisomerase II that are active against *S. pombe* strains have not been described. In this work, we show that a variety of DNA repair and damage tolerance pathways are important for the survival of *S. pombe* cells exposed to topoisomerase-targeting agents. Some pathways, notably excision repair pathways, have considerably greater effects on *S. pombe* cells than has been observed with *S. cerevisiae* mutants that are defective in homologous pathways.

MATERIALS AND METHODS

Yeast strains, plasmids, and drug treatment of *S. pombe* cells. *S. pombe* strains used in this study are shown in Table 2. Cell growth and drug treatments were performed in YES medium (5 g of yeast extract and 30 g of dextrose supplemented with 50 mg each of adenine, histidine, leucine, lysine, and uracil per liter). Logarithmically growing cultures were diluted to 2×10^6 cells/ml, and drug dissolved in dimethyl sulfoxide (DMSO) or DMSO as a solvent control was added. Aliquots were removed, diluted, and plated onto YES medium solidified with 1.5% (wt/vol) Bacto agar to determine cell viability. Plates were incubated for 3 to 4 days at 30°C before the number of CFU were counted. Relative survival values were calculated by dividing the number of colonies obtained after 8 or 24 h in the presence or absence of drug by the number of cells obtained at time 0 (the time drug was added) and multiplying by 100. All experiments were performed in triplicate at least. Results are presented \pm standard errors of the means (SEM).

TABLE 2. *S. pombe* strains used in this study and their genotypes

Strain	Genotype	Source
Fy261 (wt) ^a	<i>h⁺ ura4-D18 leu1-32 ade6-M210 can1-1</i>	ATCC ^b
1195	<i>h⁻ rad2::ura4 ade6-704 leu1032 urad4-Δ18</i>	A. Carr
1196	<i>h⁺ rad2::ura4 ade6-704 leu1032 urad4-Δ18</i>	A. Carr
1501	<i>uve1⁻ Δ(uvde1-Δ)</i>	A. Carr
942	<i>h⁺ rad13::ura4 ade6-704 leu1-32 ura4-Δ18</i>	A. Carr
1502	<i>rad2⁻ Δ rad13-Δ uve1-Δ (uvde-ΔL-2)</i>	A. Carr
1503	<i>rad2⁻ Δ rad13 Δ uve1-Δ (uvde-Δ L-5)</i>	A. Carr
1342	<i>h⁺ rhp51-ade6-704 leu1-32 ura4-Δ 18</i>	A. Carr
5094	<i>h⁻ rad22::ura4 ura4-Δ 18 arg3⁻</i>	A. Carr
5095	<i>h⁺ rhp54::ura4 ura4-Δ 18 arg3⁻</i>	A. Carr
5096	<i>h⁺ rad32::ura4 ura4-Δ 18 arg3⁻</i>	A. Carr
Rad1	<i>h⁻ rad1-1</i>	ATCC
Rad9	<i>h⁻ rad9-192</i>	ATCC
Rad8	<i>h⁻ rad8-190</i>	ATCC

^a wt, wild type.

^b ATCC, American Type Culture Collection.

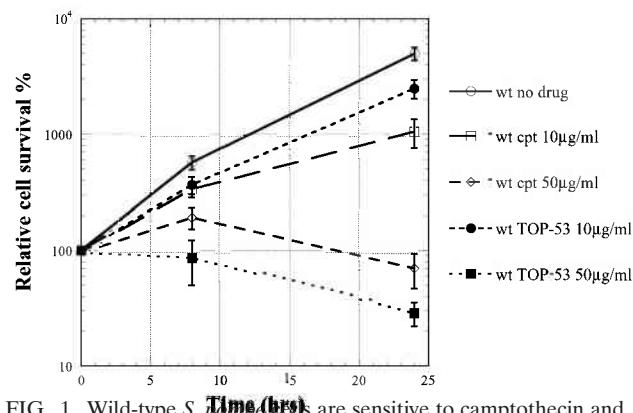


FIG. 1. Wild-type *S. pombe* (Fy261) cells are sensitive to camptothecin and TOP-53. Fy261 cells were exposed to different concentrations of camptothecin and TOP-53 for the indicated times. Aliquots were removed, and diluted samples were plated on YES medium as described in Materials and Methods. The drug concentrations used are indicated in the figure. Survival rates are expressed in percentages relative to the number of viable colonies at the time of drug addition.

The *pnm1-rad13* vector was constructed by cloning the entire *rad13* gene sequence into the *pnm1-TOPO* expression vector (Invitrogen). The *rad13* coding sequence was amplified from genomic DNA with the primers TAACCAAG GCTTACCTCG and GAGAGCAACAAAGACG. The amplified fragment was then cloned into *pnm1-TOPO* vector following the manufacturer's instructions. Transformation of *pnm1-rad13* into the *rad13*-deficient strain restored UV sensitivity to wild-type levels (data not shown).

Determination of the mlc. The minimum lethal concentration (mlc) for various drugs was determined by extrapolating cell survival at 24 h versus drug concentration curves and determining the point that gives 100% cell survival relative to the viability at $t = 0$. The mlc therefore indicates the drug concentration required to give complete growth inhibition after 24 h of exposure in YES medium.

Determination of drug sensitivity with plate assays. To assay *S. pombe* cell sensitivity to camptothecin in plates, strains were grown in liquid YES medium to an optical density at 595 nm (OD_{595}) of 0.3. The cells were then serially diluted 10-fold, and 5- μ l aliquots were spotted onto YES agar plates containing DMSO (solvent control) or YES agar plates containing 5 or 10 μ g of camptothecin/ml. The plates were grown at 30°C for 3 to 4 days and photographed.

Cell irradiation. Exponentially growing haploid cells ($OD_{550} = 0.5$) were collected by centrifugation and resuspended in a 0.1 vol of YES medium. Cell suspensions were irradiated with a ¹³⁷Cs irradiator at a dose rate of approximately 10 Gy/min. Appropriate dilutions from wild-type and mutant cells were plated on YES agar, and the survival rates were determined by counting the CFUs per plate after 3 days of incubation at 30°C.

Determination of cell cycle arrest. Delay in the cell cycle progression following exposure to hydroxyurea was detected by comparing the phenotypes of the cells with and without treatment. Cells were inoculated in YES medium and grown overnight with vigorous shaking at 30°C. With the use of a hemacytometer, the cells were normalized to a final dilution of 2×10^6 per ml and drugs were added. At 6 h after addition of 100 mM hydroxyurea or indicated concentrations of antitopoisomerase drugs, aliquots were removed and examined under the microscope at $\times 100$ magnification. Cells were photographed under polarized light with a Zeiss Axioskop2. Cells were scored as elongated when the measured length of the cells on photographs was more than 150% of the length of untreated cells.

RESULTS

Wild-type *S. pombe* strain shows sensitivity to topoisomerase-targeting agents. We first tested a wild-type strain of *S. pombe* (Fy261) for sensitivity to topoisomerase I- and topoisomerase II-targeting drugs. As shown in Fig. 1, Fy261 cells were sensitive to the topoisomerase I inhibitor camptothecin (a result consistent with previous reports) (12). We then tested whether wild-type cells were sensitive to a topoisomerase II-targeting drug. We failed to observe sensitivity to the epipodo-

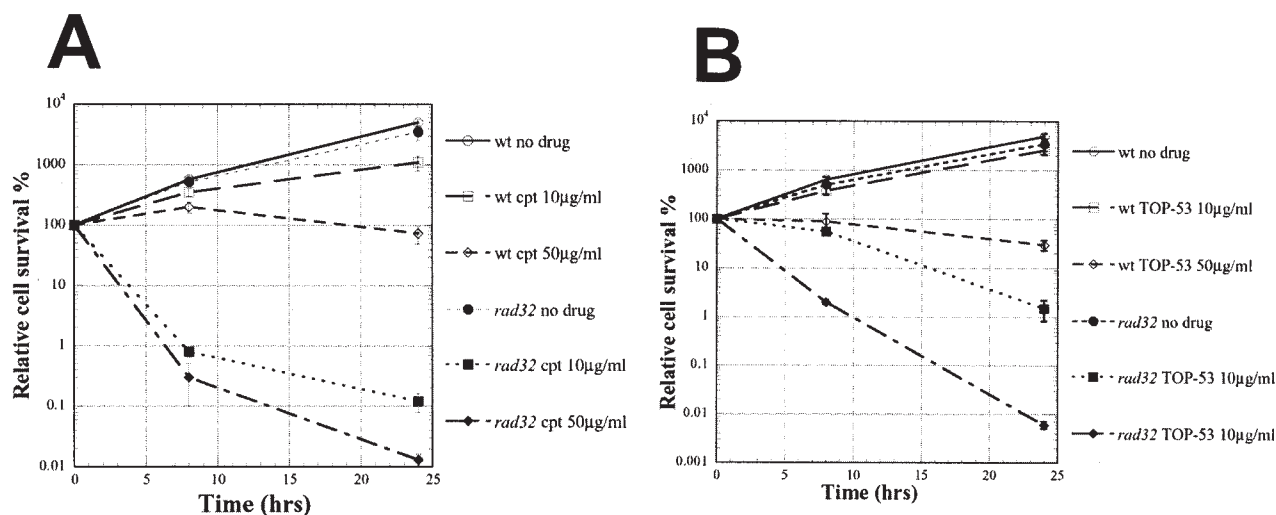


FIG. 2. Sensitivity to camptothecin and TOP-53 of *S. pombe* cells carrying *rad32* mutations. *S. pombe* cells carrying mutation in *rad32* were examined for sensitivity to camptothecin (A) or TOP-53 (B). The data for the wild-type strain FY261 are also plotted to illustrate the differences observed between the mutant and wild-type strains.

phyllotoxin etoposide or the intercalating agent amsacrine in Fy261 cells (data not shown). Fy261 cells were sensitive to a different epipodophyllotoxin, TOP-53 (Fig. 1). It is very likely that TOP-53 acts specifically on topoisomerase II, since it has been shown to specifically target topoisomerase II in *S. cerevisiae* (5, 31). Sensitivity to doxorubicin and mitoxantrone was also seen; however, these agents have mechanisms of cell killing that are independent of the presence of topoisomerase II (27).

***S. pombe* strains deficient in homologous recombination are hypersensitive to topoisomerase-targeting drugs.** We next examined the effects of topoisomerase-targeting drugs on strains carrying mutations in genes required for homologous recombination. Figure 2A shows the sensitivity to camptothecin of a strain defective in *rad32*, the *S. pombe* homolog of *MRE11* (38). Data shown in Fig. 1 for the wild-type strain Fy261 were replotted to illustrate the degree of hypersensitivity observed. Treatment of wild-type cells with 50 µg of camptothecin/ml

growth inhibitory over a 24-h exposure, with minimal cell killing. By contrast, *rad32* cells showed nearly a 4-log decrease in viable titer with a 24-h drug exposure. Exposure for shorter periods of time, or to lower concentrations of camptothecin, also resulted in substantial cell killing. The mlc of camptothecin for the *rad32*-deficient strain was approximately 0.5 µg/ml compared to 50 µg/ml for the wild-type strain (data not shown). Similarly, treatment of *rad32* cells with TOP-53 resulted in substantial cell killing (Fig. 2B) compared to the results obtained with wild-type cells, with an mlc of 2 to 5 µg of TOP-53/ml for *rad32* cells compared to an mlc of approximately 30 to 40 µg of TOP-53/ml for the wild-type strain.

The role for homologous recombination in sensitivity to topoisomerase-targeting agents was confirmed by assessing the sensitivity of mutants with defects in other genes in this pathway. Figure 3A shows the sensitivity of *rad22A* and *rhp54* mutants to camptothecin, and Fig. 3B shows the results obtained

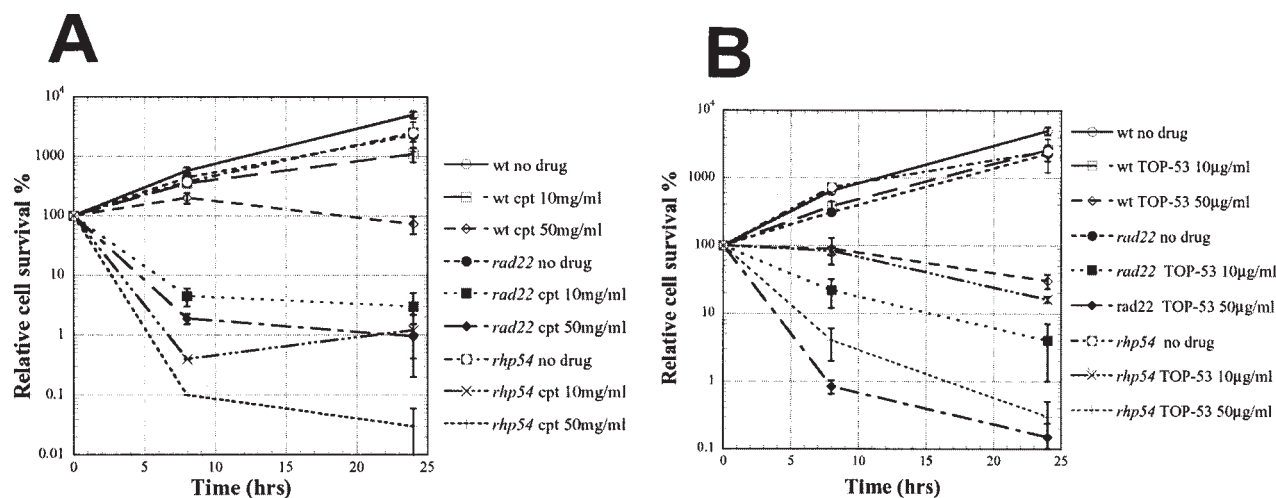


FIG. 3. Sensitivity to camptothecin and TOP-53 of *S. pombe* cells carrying *rad22A* or *rhp54* mutations. *S. pombe* cells carrying mutations in *rad22A* or *rhp54* were examined for sensitivity to camptothecin (A) or TOP-53 (B). The data for the wild-type strain FY261 are also plotted to illustrate the differences observed between the mutant and wild-type strains.

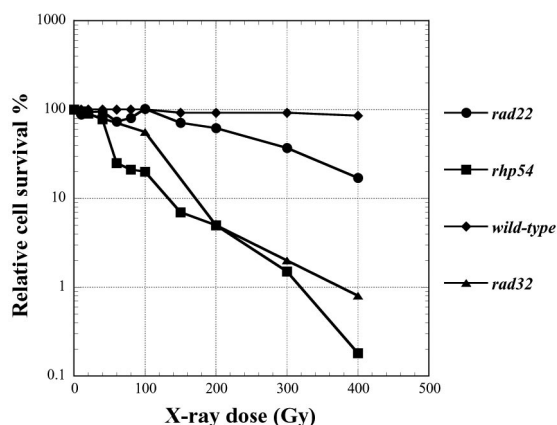


FIG. 4. Comparative sensitivities of *rad22A* and *rhp54* cells to ionizing radiation. *S. pombe* cells carrying mutations in *rad22A* or *rhp54* were examined for sensitivity to ionizing radiation. Cell survival of the mutants was compared to that of Rad^+ (Fy261) cells.

with TOP-53. These genes are homologs of *RAD52* and *RAD54*, respectively (21, 22). As was seen with *rad32* mutants, *rad22A* and *rhp54* mutants showed enhanced sensitivity to both camptothecin and TOP-53. While there was a slight difference in the survival rates of the *rad22A* mutant at high camptothecin concentrations compared to those of the *rhp54* mutant, the mlc values of camptothecin for the *rad32*, *rhp54*, and *rad22* strains were all determined to be 0.5 $\mu\text{g/ml}$ (data not shown). Sensitivity of all three recombination-deficient strains was also comparable to that for TOP-53 (Fig. 3B). We also examined the sensitivity of an *rhp51* strain to camptothecin and TOP-53 and observed no significant difference from the results seen with the other recombination-deficient mutants described here (data not shown).

The results obtained for sensitivity of the *rad22A* mutant strain to camptothecin and TOP-53 were of particular interest, since this allele had been characterized as having only slight sensitivity to ionizing radiation compared to those of other recombination-deficient mutants such as *rad32* and *rhp54*. Recent results indicated that a full deletion of the *rad22A* gene confers the same sensitivity to ionizing radiation as mutations in genes such as *rhp51* (40, 41). We confirmed that the *rad22A* allele used here has only a slight sensitivity to ionizing radiation compared to that of strains bearing mutations in *rad32* or *rhp54* (Fig. 4). Since our results demonstrate that this *rad22A* allele has a high level of sensitivity to topoisomerase-targeting agents, those results suggest that this allele is completely defective in repair arising from this type of DNA damage.

Excision repair mutants of *S. pombe* are sensitive to camptothecin and TOP-53. Since the “adduct” formed by topoisomerase-targeting drugs includes both DNA strand breaks and protein covalently bound to DNA, we hypothesized that other repair pathways would be required to effect complete repair of topoisomerase-mediated DNA damage. *S. pombe* cells have two distinct excision repair pathways. One pathway is homologous to the yeast RAD1/10 pathway that introduces DNA cuts on both sides of a DNA lesion (6). *S. pombe* cells have a second pathway that is shared with higher eukaryotes but is apparently absent from *S. cerevisiae* (1, 4, 13). This alternate pathway uses a damage-specific endonuclease (Uve1p) that

TABLE 3. Sensitivity of excision repair-defective strains to camptothecin^a

Strain	% Survival after 24 h (no drug)	% Survival after treatment for 24 h with camptothecin (10 $\mu\text{g/ml}$)	% Survival after treatment for 24 h with camptothecin (50 $\mu\text{g/ml}$)
Fy261 (wt)	5,000 \pm 650	1,100 \pm 300	73 \pm 24
1195 (<i>rad2</i>)	4,400 \pm 620	340 \pm 94	34 \pm 7
942 (<i>rad13</i>)	6,100 \pm 950	820 \pm 50	43 \pm 25
1501 (<i>uve1</i>)	6,000 \pm 2,000	1,900 \pm 340	820 \pm 50

^a Strains were exposed to camptothecin in liquid culture as described in Materials and Methods and plated to determine viable titers. The results shown are the relative survival rates [(survival at 24 h)/(initial viable titer at the time of drug addition) \times 100] \pm SEM.

^b wt, wild type.

introduces a cut immediately 5' of sites of DNA damage. In addition to the requirement for Uve1, this alternate pathway also requires the *S. pombe* homolog of the flap endonuclease FEN-1. A flap endonuclease homolog is present in *S. cerevisiae* and is encoded by the *RAD27* gene.

We examined the sensitivity of cells defective in the canonical excision repair pathway (*rad13*[−]) as well as that of cells deficient in the alternate pathway. The results determined after 24 h of exposure to camptothecin are shown in Table 3. A slight sensitivity to camptothecin was seen in *rad13*[−] and *rad2*[−] cells, while *uve1*[−] cells had enhanced resistance to camptothecin compared to wild-type cells.

Because the enhanced sensitivity to camptothecin was relatively slight, we carried out two additional experiments to verify that the sensitivity was due to a defect in excision repair. First, we constructed a plasmid carrying the wild-type *rad13* gene. Introduction of *rad13*⁺ complemented both the UV sensitivity and the camptothecin sensitivity of strain 941 (data not shown).

We also applied an alternate approach for measurement of the camptothecin sensitivity of the excision-deficient cells. Serial dilutions of cultures were spotted on plates containing 10 μg of camptothecin/ml. As shown in Fig. 5 both strain 1195

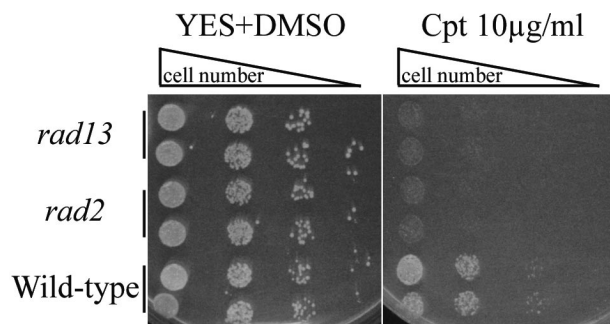


FIG. 5. Sensitivity of excision repair-defective strains to camptothecin determined using drug-containing plates. An excision repair-defective strain defective in the *rad13* gene and a strain lacking the flap endonuclease *rad2* were examined for camptothecin sensitivity. Cells were pregrown in liquid YES growth medium to an OD_{595} of 0.3 and serially diluted 10-fold, and 5 μl -aliquots of the diluted samples were spotted onto YES agar plates with or without camptothecin (camptothecin-containing plates included 10 μg of camptothecin/ml). The plates were incubated at 30°C for 3 days and photographed under white light.

TABLE 4. Sensitivity of excision repair-defective strains to TOP-53^a

Strain	Survival after 24 h (no drug)	Survival after treatment for 24 h with TOP-53 (50 µg/ml)
Fy261 (wt) ^b	5,000 ± 650	30 ± 7
1195 (<i>rad2</i>)	5,200 ± 942	8 ± 3
1501 (<i>uve1</i>)	6,300 ± 330	3 ± 0.4
942 (<i>rad13</i>)	8,000 ± 820	2 ± 1

^a Strains were exposed to TOP-53 in liquid culture as described in Materials and Methods and plated to determine viable titers. The results shown are the relative survival rates [(survival at 24 h)/(initial viable titer at the time of drug addition) × 100] ± SEM.

^b wt, wild type.

(*rad2*) and strain 941 (*rad13*) cells failed to grow on plates containing camptothecin. The relatively high level of sensitivity seen on plates with camptothecin arises in part because the assay using camptothecin-containing plates does not distinguish between strong growth inhibition and cell killing. Nonetheless, the results seen with the plate assay are consistent with the conclusion that excision repair functions play a role in sensitivity to camptothecin in *S. pombe* cells.

We also assessed the sensitivity of excision repair-deficient cells to TOP-53. The results of a 24-h exposure are shown in Table 4. Enhanced killing by TOP-53 was seen with all three mutant cell lines examined. Cells deficient in canonical excision repair had a more than 10-fold-lower survival rate than wild-type cells. Both *rad2*-deficient and *uve1*-deficient cells also showed reduced survival compared to wild-type cells. Due to the limited availability of TOP-53, we were unable to apply the plate assay that we used for camptothecin. Nonetheless, the rates of survival following a 24-h exposure to TOP-53 were significantly different for all three mutant strains compared to those seen with the wild-type cells. It is noteworthy that there is a clear difference in the response of the *uve1*[−] cells to camptothecin versus that seen with TOP-53. Whereas the *uve1*[−] cells were slightly hypersensitive to TOP-53, they had

resistance to camptothecin. This result suggests that Uve1p might be able to act on topoisomerase I covalent complexes but that the activity of this protein diminishes rather than enhances repair.

Checkpoint-defective mutants of *S. pombe* show hypersensitivity to topoisomerase-targeting drugs. An important response to DNA damage or inhibition of replication fork progression is the induction of cell cycle arrest by checkpoint functions. A particular strength of the *S. pombe* system is the very-well-characterized genetics of checkpoint responses (7, 34). Previous work with *S. pombe* by Walworth and colleagues showed that checkpoint defects increased sensitivity to camptothecin (43), a result in agreement with findings obtained with *S. cerevisiae* (20, 35). However, *S. cerevisiae* cells lacking *rad9* were not more sensitive to topoisomerase II-targeting drugs than wild-type cells (33). We first confirmed that checkpoint genes affect sensitivity to camptothecin. Figure 6A shows the sensitivity of *S. pombe* cells lacking *rad9* compared to that of wild-type cells. The mlc of camptothecin for *rad9* mutants was approximately 1 µg/ml (data not shown). The degree of sensitivity seen for *S. pombe rad1* mutants (data not shown) was similar to that observed with the *rad9* mutant whose results are shown in Fig. 6A. Both *rad1* and *rad9* mutants also displayed hypersensitivity to TOP-53. The results of investigations of the sensitivity of *rad9* mutants to TOP-53 are shown in Fig. 6B. The mlc of *rad9* mutants to TOP-53 was 5 µg/ml, and a similar value was also obtained for *rad1* mutants (data not shown). These findings indicate that a lack of DNA damage checkpoints enhances sensitivity to both topoisomerase I- and topoisomerase II-targeting agents. The results shown below also demonstrate that these drugs are effective at inducing cell cycle arrest and that the ability to carry out cell cycle arrest is abolished in *rad1* and *rad9* mutants.

Hypersensitivity of *RAD8* mutant cells to topoisomerase-targeting drugs. In addition to its utility for comparisons of known DNA repair functions, another strength of an alternate genetic system for studying topoisomerase-targeting agents is

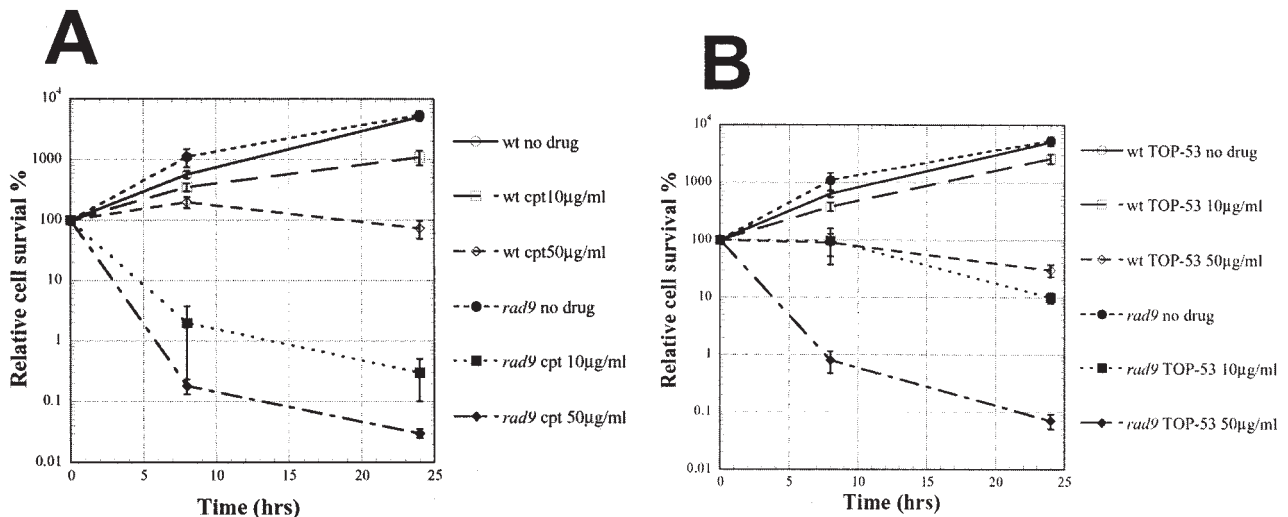


FIG. 6. Checkpoint defects lead to enhanced sensitivity to both topoisomerase I- and topoisomerase II-targeting drugs. *S. pombe* cells carrying a mutation in *rad9* were examined for sensitivity to camptothecin (A) or TOP-53 (B). The data for the wild-type strain FY261 are also plotted to illustrate the differences observed between the mutant and wild-type strains.

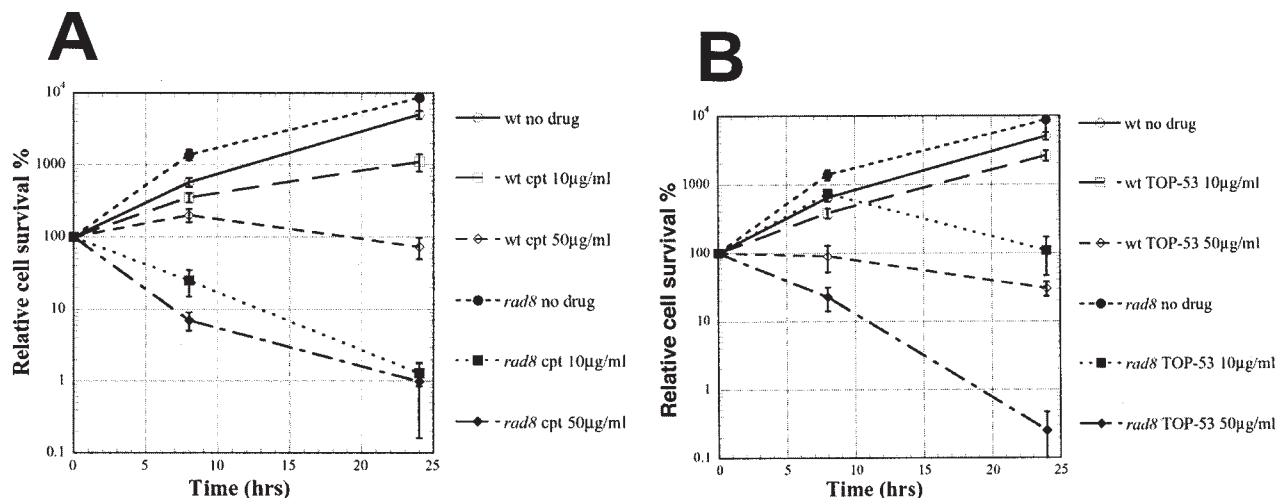


FIG. 7. Mutations in the *rad8* gene enhance topoisomerase-targeting drug sensitivity. *S. pombe* cells carrying a mutation in *rad8* were examined for sensitivity to camptothecin (A) or TOP-53 (B). The data for the wild-type strain Fy261 are also plotted to illustrate the differences observed between the mutant and wild-type strains.

the ability to identify genes that are apparently absent from other systems. The results described above that were obtained with the alternate excision repair pathway demonstrate the usefulness of *S. pombe* in studying topoisomerase-targeting drugs. As an additional example, we examined the sensitivity conferred by *S. pombe rad8* mutants. *S. pombe rad8* cells show enhanced sensitivity to both UV and ionizing radiation (10). As shown in Fig. 7A, *rad8* mutants also have enhanced sensitivity to camptothecin. Similarly, *rad8* mutants are also hypersensitive to TOP-53 (Fig. 7B). The results of the investigations involving the *rad8* mutant strain were obtained with a strain carrying a point mutation. To verify the importance of the *rad8* gene, we also constructed strains lacking the *rad8* gene. The levels of sensitivity to both camptothecin and TOP-53 (data not shown) were identical to the results shown in Fig. 7. Since the phenotype of *rad8* mutants has not been analyzed in detail, we tested whether the *rad8* mutants carried out a normal checkpoint arrest in response to the presence of hydroxyurea or of topoisomerase-targeting agents.

Wild-type, *rad1*, *rad9*, or *rad8* cells were grown overnight, diluted, and then exposed to a solvent control, hydroxyurea, camptothecin, or TOP-53. Cell populations treated with DMSO showed no abnormalities, although *rad8* cells were somewhat elongated (Fig. 8). After 6 h in hydroxyurea, wild-type cells became substantially elongated. The results of investigations of the percentage of cells that were elongated are shown in Table 5. Similar numbers of elongated cells were observed when wild-type cells were treated with either 50 µg of camptothecin/ml or 50 µg of TOP-53/ml. By contrast, *rad1* and *rad9* cells showed no increase in the percentage of elongated cells and closely resembled cells treated with DMSO alone. Like the wild-type cells, drug-treated *rad8* cells were also elongated compared to the cells without any treatment. Although the percentage of elongated cells was not different from that of the wild type, the morphology of TOP-53-treated *rad8*-deficient cells appeared more extreme than that of wild-type cells treated with TOP-53. This result suggests that *rad8* cells grown in the absence of exogenous DNA-damaging agents might have a partial defect

in DNA metabolism that is exacerbated by TOP-53. Possible functions of the *rad8* gene in sensitivity to topoisomerase-targeting agents are described in the Discussion.

DISCUSSION

It is well established that topoisomerase inhibitors that stabilize enzyme:DNA covalent complexes are cytotoxic primarily because they generate DNA damage (26, 42). The primary damage consists of the enzyme covalently bound to DNA. Drug-stabilized covalent complexes are reversible when the drug is removed, but they can be converted to irreversible damage by collision with enzymes tracking along DNA, such as DNA polymerases. Many topoisomerase-targeting drugs are superb probes for DNA repair functions, since they have been shown to be highly specific for their targets. For example, camptothecins have been shown to be highly specific for topoisomerase I (12, 24). Similarly, epipodophyllotoxins and other topoisomerase II-targeting agents have also been shown to be highly specific for their targets (5, 17, 29). Since both topoisomerase I and topoisomerase II are targets for active anti-cancer agents, understanding cellular responses to these agents also lends insight into their appropriate clinical use.

Previous studies have elaborated the yeast *S. cerevisiae* as a genetically tractable model system for studying topoisomerase-targeting agents (24, 27). The goal of the present work has been to develop a parallel system using the fission yeast *S. pombe*. Our initial goals were to demonstrate whether conclusions drawn from studies with budding yeast were applicable to other eukaryotic organisms. We also wished to examine repair functions that are found in fission yeast but that have not been identified in budding yeast or that may have subtly different biochemical roles. As was observed previously, we found that *S. pombe* cells have sensitivity to camptothecin. We examined several different topoisomerase II agents targeting wild-type *S. pombe* cells and found that the epipodophyllotoxin TOP-53 is active against wild-type *S. pombe* cells. Experiments with drug-resistant *top2* mutants of *S. cerevisiae* clearly showed that

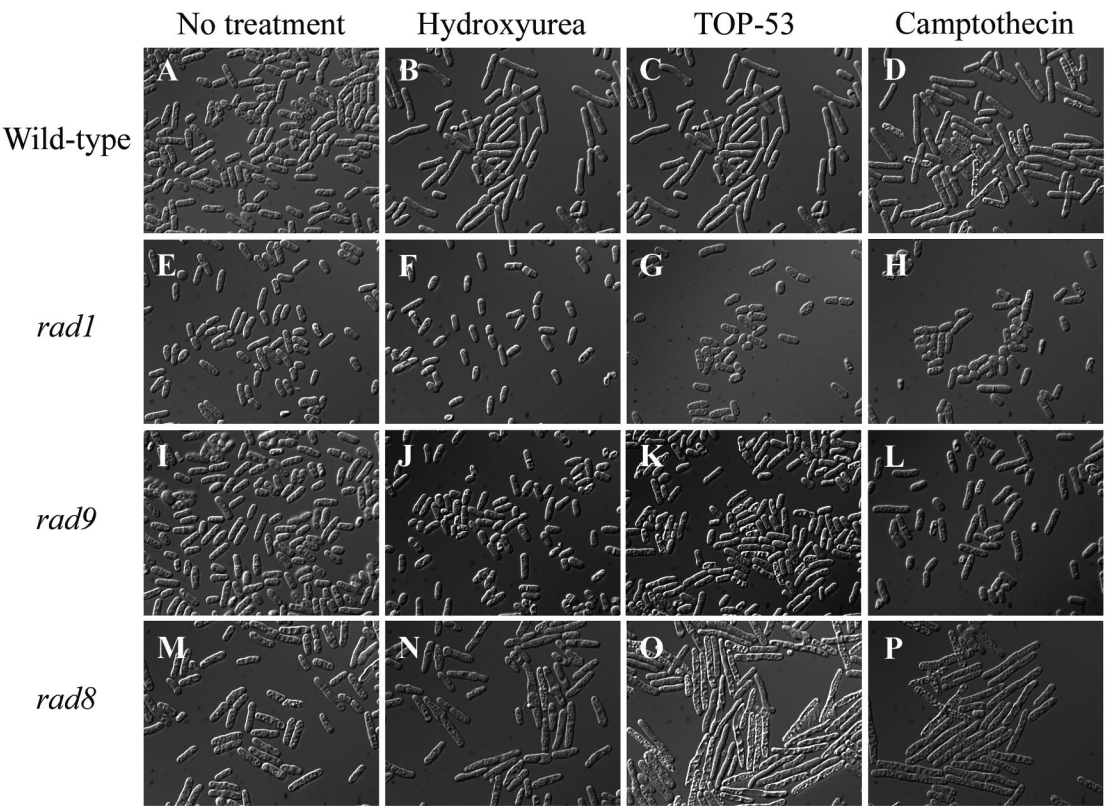


FIG. 8. Visualization of checkpoint mutants of *S. pombe* exposed to topoisomerase-targeting agents. Cells were inoculated in YES medium and grown overnight with vigorous shaking at 30°C. After the cells were counted in a hemacytometer and diluted to 1×10^6 to 2×10^6 cells per ml, drugs were added as indicated. After 6 h, aliquots were placed on glass slides and examined under a microscope. Strains: wild type, panels A, B, C, and D; *rad1*, panels E, F, G, and H; *rad9*, panels I, J, K, and L; and *rad8*, panels M, N, O, and P. The results seen after 6 h of no drug treatment (panels A, E, I, and M), 6 h in 100 mM hydroxyurea (panels B, F, J, and N), 6 h of treatment with 50 μ g of TOP-53 per ml (panels C, G, K, and O), and 6 h of treatment with 50 μ g of camptothecin per ml (panels D, H, L, and P) are shown. *S. pombe rad8* cells were slightly larger on average but otherwise indistinguishable from those of the other strains under all conditions indicated.

TOP-53 specifically targets topoisomerase II and is not active against other possible cytotoxic targets (5).

One important function that we examined in this work is the role of genes required for recombinational repair in cell survival following treatment with topoisomerase-targeting agents. As noted in Results, some disruption alleles of the *rad22A^{Sp}* gene, a homolog of the *S. cerevisiae RAD52* gene, displayed less sensitivity to ionizing radiation than those of other mutants deficient in recombinational repair. Although such *rad22A⁻* cells are less sensitive to ionizing radiation than other *S. pombe* recombination-deficient mutant cells, they show levels of sensitivity to both the topoisomerase I-targeting agent camptothec-

in and the topoisomerase II-targeting epipodophyllotoxin TOP-53 similar to those of other mutants deficient in genes encoding recombination repair factors, such as *rad32* and *rhp51* (*S. pombe* homologs of *S. cerevisiae MRE11* and *RAD51*, respectively). It is thought that double-strand breaks are the most important lesions induced by ionizing radiation that require recombinational repair (3). Our results suggest that not all double-strand breaks are equivalent and that the breaks arising from topoisomerases have an enhanced requirement for *rad22A*. This might occur because of the association of DNA replication forks with the generation of double-strand breaks induced by topoisomerase inhibitors. A double-strand

TABLE 5. Cell cycle arrest phenotype of *S. pombe* wild-type, *rad1*, *rad8*, and *rad9* strains following treatment with topoisomerase poisons

Strain	Results after treatment with:							
	No drug		Hydroxyurea		Camptothecin		TOP-53	
	Total no. of cells	% Elongated cells	Total no. of cells	% Elongated cells	Total no. of cells	% Elongated cells	Total no. of cells	% Elongated cells
FY261	798	0	563	42	536	47	627	46
<i>rad1</i>	612	0.5	402	6	526	0.8	730	2.6
<i>rad9</i>	793	1.7	703	1.3	259	1.5	700	2.8
<i>rad8^a</i>	493	3.9	302	27	293	44	366	59

^a The average size of *rad8* mutant cells in the absence of drug was slightly larger than that of wild-type, *rad1*, or *rad9* cells.

break occurring at a replication fork may have a stringent requirement for *rad22A*, while double-strand breaks occurring far from replication forks (as would occur for most breaks arising from ionizing radiation) may have a lesser requirement for *rad22A*. Alternately, topoisomerase-targeting drugs may be a very efficient generator of double-strand breaks, leading to a requirement for high levels of *rad22A* protein for cell survival.

The work described here also lends insights into the repair of the covalent protein DNA adducts arising from topoisomerase-mediated DNA damage. Clearly, defects in excision repair do not sensitize cells to the same extent as mutations in genes required for recombinational repair or required for DNA damage checkpoints. This suggests that the proteins involved in excision repair represent one of several protein functions that are able to process covalent protein DNA adducts. It is also possible that excision repair proteins do not function in *S. pombe* to remove protein covalently bound to DNA but are instead required for processing DNA strands, allowing them to be substrates for homologous recombination or other break repair pathways. However, Vance and Wilson have argued that the Rad1/Rad10 nuclease functions as an alternate pathway for the removal of topoisomerase I trapped by camptothecin (39). Their argument is based on the observation that *S. cerevisiae* mutants lacking *rad1* or *rad10* do not display camptothecin sensitivity but that mutants defective in both *rad1* (or *rad10*) and *tdp1* (a gene encoding a tyrosine phosphodiesterase that removes peptides bound 3' to DNA) have substantial camptothecin sensitivity. We have also constructed *S. pombe* mutants defective in the tyrosine phosphodiesterase that can remove peptides bound to the 3' end of DNA. As was observed by Nash and colleagues in investigations of *S. cerevisiae* (35), mutations in the *S. pombe* homolog of *tdp1* fail to greatly increase the sensitivity of cells to camptothecin or other DNA-damaging agents. It is unlikely that canonical excision repair functions act on topoisomerase DNA covalent adducts, due to the relatively large size of the topoisomerase proteins. Further experiments with purified excision repair components will be needed to clarify how these proteins can process topoisomerase DNA covalent complexes.

Although we have demonstrated that *S. pombe* has considerable advantages as a genetic system for studying topoisomerase inhibitors, there are several areas that can be examined to greatly enhance the utility of this organism for studying anticancer agents. As described in Results, wild-type *S. pombe* cells did not show sensitivity to etoposide or mAMSA {N-[4-(9-acridinylamino)-3-methoxyphenyl]methanesulphonanilide}, presumably due to a lack of significant accumulation of these agents. To overcome this problem, the construction of strains that carry mutations in genes enhancing drug uptake or reducing drug efflux would be useful. Good candidates from studies in *S. cerevisiae* include genes such as *ERG6*, a gene required for C-24 methylation in the biosynthesis of ergosterol (14), and *PDR5*, a transmembrane ATP binding transport protein involved in the efflux of a variety of small molecules (2). Proteins that may be involved in drug efflux have already been identified in *S. pombe* (23).

A major advantage of the *S. pombe* system is the identification of novel proteins required for surviving topoisomerase-mediated damage. These may include proteins such as Uve1 that are absent in other systems and proteins such as Rad8 that

are not obviously equivalent to proteins in other organisms. The *rad8* gene has homology to the *S. cerevisiae* *RAD5* gene, but it has not yet been determined whether its roles in repair are similar (10). Since the functions of the *S. cerevisiae* *RAD5* gene are also poorly understood, more detailed experiments with both organisms will be needed to enhance understanding of the roles of both genes in repairing topoisomerase-mediated damage. Preliminary experiments using *S. cerevisiae* failed to show enhanced sensitivity of *rad5* mutants to either camptothecin or topoisomerase II-targeting agents (J. L. Nitiss, unpublished results), suggesting that if *rad8* is the *S. pombe* homolog of *RAD5*, it plays a more critical role in surviving topoisomerase-mediated DNA damage.

The results obtained with *uve1* mutants are particularly intriguing. Although *uve1* mutants are slightly more sensitive to TOP-53 than wild-type cells, they are markedly resistant to camptothecin. This result suggests that some repair pathways might initiate repair reactions at topoisomerase covalent complexes and process the lesion in a way that makes it more difficult to repair. The agent ecteinascidin 743 has recently been suggested to be an agent whose toxicity is increased by the action of excision repair pathways. Our results suggest that this might also be the case for some topoisomerase-mediated DNA damage. Since the Uve1 protein has recently been purified, it will be possible to assess whether topoisomerase DNA adducts are substrates for this protein.

While the experiments described in this paper all deal with a lower eukaryotic model system, we postulate that our observations will also be relevant for understanding the action of topoisomerase-targeting agents in higher eukaryotes. As indicated above, DNA topoisomerases are important clinical targets for a wide range of anticancer agents. The importance of several different DNA repair pathways for cell survival following treatment with topoisomerase-targeting agents (as we have observed in this work) may indicate why combinations of these drugs with other DNA-damaging agents frequently result in synergistic cytotoxicity. An understanding of how these pathways participate in repairing topoisomerase-mediated DNA damage will not only illuminate the biochemical pathways but may also contribute to the clinical utility of these agents.

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