Roles of nonhomologous end-joining pathways in surviving topoisomerase II–mediated DNA damage

Mobeen Malik, Karin C. Nitiss, Vanessa Enriquez-Rios, and John L. Nitiss
Department of Molecular Pharmacology, St. Jude Children’s Research Hospital, Memphis, Tennessee

Abstract
Topoisomerase II is a target for clinically active anticancer drugs. Drugs targeting these enzymes act by preventing the religation of enzyme-DNA covalent complexes leading to protein-DNA adducts that include single- and double-strand breaks. In mammalian cells, nonhomologous repair pathways are critical for repairing topoisomerase II–mediated DNA damage. Because topoisomerase II–targeting agents, such as etoposide, can also induce chromosomal translocations that can lead to secondary malignancies, understanding nonhomologous repair of topoisomerase II–mediated DNA damage may help to define strategies that limit this critical side effect on an important class of anticancer agents. Using Saccharomyces cerevisiae as a model eukaryote, we have determined the contribution of genes required for nonhomologous end-joining (NHEJ) for repairing DNA damage arising from treatment with topoisomerase II poisons, such as etoposide and 4-[(9-acridinylamino)methanesulfonyl]-m-anisidide (mAMSA). To increase cellular sensitivity to topoisomerase II poisons, we overexpressed either wild-type or drug-hypersensitive alleles of yeast topoisomerase II. Using this approach, we found that yku70 (hdf1), yku80 (hdf2), and other genes required for NHEJ were important for cell survival following exposure to etoposide. The clearest increase in sensitivity was observed with cells overexpressing an etoposide-hypersensitive allele of TOP2 (Ser740Trp). Hypersensitivity was also seen in some end-joining defective mutant exposed to the intercalating agent mAMSA, although the increase in sensitivity was less pronounced. To confirm that the increase in sensitivity was not solely due to the elevated expression of TOP2 or due to specific effects of the drug-hypersensitive TOP2 alleles, we also found that deletion of genes required for NHEJ increased the sensitivity of rad52 deletions to both etoposide and mAMSA. Taken together, these results show a clear role for NHEJ in the repair of DNA damage induced by topoisomerase II–targeting agents and suggest that this pathway may participate in translocations generated by drugs, such as etoposide.

Introduction
DNA topoisomerases are essential enzymes required to maintain normal DNA topology in cells (reviewed in refs. 1, 2). The reaction cycle of topoisomerase II consists of DNA binding, DNA cleavage, DNA strand passage, and religation of the cleaved DNA (3, 4). DNA cleavage involves formation of a reversible intermediate consisting of an active site tyrosine residue that forms a phosphotyrosyl linkage with DNA. Agents targeting DNA topoisomerase II, which include important anticancer and antibacterial agents, interfere with the normal enzyme reaction cycle resulting in accumulation of DNA strand breaks with the enzyme covalently bound to DNA. These covalent complexes are reversible if drug is removed but can be converted into cytotoxic DNA damage upon collision between the complexes and replication forks or other DNA-tracking proteins.

Consistent with generation of DNA strand breaks, homologous recombination pathways are important for cell survival following exposure to topoisomerase II poisons (5). Saccharomyces cerevisiae cells defective in rad52 as well as strains with defects in other recombination repair genes, such as rad50, mre11, and rad54 mutants, are hypersensitive to topoisomerase II poisons (6, 7). Similar results have been observed in fission yeast mutants that are defective in DNA repair by homologous recombination (8).

Other pathways for carrying out double-strand break (DSB) repair do not depend on homologous DNA as a template for repair. A critical pathway for nonhomologous repair of DSBs is the nonhomologous end-joining (NHEJ) pathway (9, 10). The essential components of NHEJ are broadly conserved among eukaryotes. Yeast and mammalian cells encode similar critical components, including the heterodimeric DNA-binding protein that is composed of Ku70 and Ku80 as well as a specialized DNA ligase termed ligase IV (reviewed in refs. 9, 11–13). There are clear differences in the components between yeast and mammalian cells; notably, yeast lacks the DNA-dependent protein kinase that plays a key role in many of the NHEJ reactions that occur in mammalian cells.

The relative importance of NHEJ in yeast cells and metazoan organisms in repairing DNA DSBs differs...
considerably. In mammalian cells, loss of NHEJ by mutations in Ku70, Ku80, or DNA ligase IV all lead to hypersensitivity to ionizing radiation (13, 14). By contrast, loss of the NHEJ pathway in yeast does not result in sensitivity to DNA-damaging agents, such as ionizing radiation (15–17), although slight sensitivity to some agents, such as bleomycin or methylmethane sulfonate, has been reported (18, 19). In yeast, combining mutations conferring deficiency in NHEJ with mutations in genes required for repair by homologous recombination results in synergistic sensitivity to ionizing radiation (13, 17, 20). This result has led to a model where NHEJ is a secondary pathway for repair of DSBs in yeast. Nonetheless, the NHEJ pathway is essential in yeast for the repair of DSBs arising from the ectopic expression of restriction endonucleases or for the repair of endonuclease-induced DSBs when the break occurs in cells lacking a homolog as a template for repair (9, 21, 22).

Mammalian cells defective in NHEJ have been shown to have high levels of etoposide sensitivity, although interestingly mutants lacking DNA-dependent protein kinase are less sensitive than mutants lacking Ku70 (23, 24). In derivatives of the chicken DT40 cell line, cells lacking ligase IV or Ku70 are extremely sensitive to etoposide, whereas cells defective in homologous recombination are not as sensitive as end-joining mutants (14, 25). Recent small interfering RNA experiments in mammalian cells are consistent with Ku70 or Ku80 playing major roles in etoposide sensitivity (26).

In nonhomologous repair pathways, DNA ends are joined with little or no base pairing at the junction; therefore, the repaired chromosome may carry insertions or deletions. Additionally, nonhomologous repair pathways have the potential to join sequences on nonhomologous chromosomes leading to chromosomal translocations (27). Etoposide and other topoisomerase II poisons can lead to secondary malignancies by the induction of specific chromosome translocations (28, 29). Thus, repair of damage by NHEJ may play a role in the induction of translocations by topoisomerase II–targeting agents.

We have taken advantage of the ability to enhance the sensitivity of repair-proficient yeast cells using overexpression of either wild-type or drug-hypersensitive alleles of yeast topoisomerase II to examine the role that NHEJ plays in repairing topoisomerase II–mediated DNA damage. In a previous report, a slight increase in sensitivity was seen with yeast cells expressing an etoposide-hypersensitive allele of yeast topoisomerase II (7). In this article, we examine a series of different deletion strains using overexpression of either etoposide-hypersensitive or 4’-(9-acridinylamino)-methanesulfon-m-anisidide (mAMSA)–hypersensitive alleles to test whether NHEJ is involved in repairing topoisomerase II–mediated DNA damage. We show that NHEJ proteins participate in protecting cells from topoisomerase-mediated damage even in cells that are proficient in homologous recombination. Our results indicate that topoisomerase II can generate DNA damage that requires the NHEJ pathway for repair.

Materials and Methods

**Yeast Strains and Plasmids**

*S. cerevisiae* strains generated for this study were derivatives of JN362a (*MATa ura3-52 leu2 trp1 his7 ade1-2 IS2; ref. 30). For the construction of *Aku70::LEU2*, the wild-type gene was amplified from genomic yeast DNA (Promega, Milwaukee, WI). The PCR product was cloned into the plasmid pCR2.1 (Invitrogen, Carlsbad, CA) using the TA cloning kit (Invitrogen). The plasmid pCR2.1KU70 was then digested with restriction enzymes to delete an internal fragment of the open reading frame followed by ligation of the entire *LEU2* gene to interrupt the coding sequence. The *Aku70::LEU2* fragment was then PCR amplified and transformed in JN362a by selection for growth in the absence of leucine. The gene replacement was confirmed by PCR of genomic DNA using primers flanking the respective gene sequences and restriction enzyme digestion. The *Aku80* strain was also constructed following the same procedure as described for JN362a *Aku70::LEU2*, except that *URA3* was used as the selective marker. After verification that the genomic *yka80* locus was disrupted, the *URA3* gene used to disrupt *ku80* was “recycled” by transformation with an *ura3* allele carrying an internal deletion. The internal deletion of *URA3* was obtained by cloning the entire *URA3* gene into T7Blue (Novagen, La Jolla, CA). The resulting plasmid was digested with EcoRV and *StuI* and then ligated with T4 DNA ligase. The *ura3* allele, carrying the EcoRV/*StuI* deletion, was amplified by PCR and transformed into JN362a *Ayku80::URA3* with selection for 5-fluoro-orotic acid resistance (31). The resulting strain carried a partial deletion of *ura3* disrupting *ku80*. The presence of the correct disruption was confirmed by demonstration of uracil auxotrophy and by PCR. For the construction of *Adnl4*, *Alif1*, *Anej1*, and *Arad52* strains in JN362a background, derivatives of BY4741 (*MATa hisA1 leu2A0 lys2A0 ura3A0; ref. 32) carrying precise deletions of the open reading frames of *DNL4*, *LIF1*, *NEJ1*, and *RAD52* were obtained from Open Biosystems (Huntsville, AL). The *Anej1::KANMX4*, *Alif1::KANMX4*, *Adnl4::KANMX4*, and *Arad52::KANMX4* alleles were PCR amplified (primer sequences used for PCR amplification are available upon request). The PCR-amplified alleles were transformed into JN362a by one-step gene disruption procedure (33). Selection for gene disruptions with the KANMX4 alleles was carried out after 2 days of growth on rich medium followed by plating to YPDA plates containing 70 μg/mL G418 (Life Technologies, St. Paul, MN). Gene replacements were confirmed by PCR of genomic DNA using primers flanking the respective gene sequence and an internal sequence within KANMX4. Yeast genomic DNA was prepared by using Y-Der Yeast DNA Extraction Reagent kit (Pierce, Rockford, IL).

To construct strains carrying mutations in both NHEJ and *rad52* (JN362a *Aku70/Arad52* and JN362a *Anej1/Arad52*), the strains JN362a *Aku70::LEU2* and JN362a *Anej1::KANMX4* were converted to *rad52* by one-step disruption using either pSM20, which carries a *LEU2* disruption of the *RAD52*
gene, or a KANMX4 disruption of RAD52 obtained as described above (34). The transformants were verified using the procedures described above and also scored for sensitivity to 0.017% methylmethane sulfonate. To construct strains carrying mutations in both NHEJ and rad1, JN362a or JN362a Δku70::LEU2 cells were converted to Arad1 using a Arad1::URA3 cassette. Transformants were verified as described above and also scored for sensitivity to UV light.

To obtain strains overexpressing wild-type yeast topoisomerase II, the appropriate strains were transformed with pDED1TOP2 (30). For expression of drug-hypersensitive alleles of TOP2, the strains were transformed with plasmids pDED1TOP2S740W (35) or pDED1TOP2T744P (36), which carry the etoposide-hypersensitive allele Ser²⁴⁶Trp or the mAMSA-hypersensitive allele Thr²⁴⁴Pro.

**Determination of Drug Sensitivity**

Cell growth and drug treatments were done in YPDA medium (10 g yeast extract (Difco, Tucker, GA), 20 g Bactopeptone (Difco), 20 g glucose, and 10 mg adenine sulfate/L) or in SD-ura medium as described previously (30). Logarithmically growing cultures were diluted to 2 × 10⁶ cells/mL, and drug dissolved in DMSO or DMSO as solvent control was added. Aliquots were removed, diluted, and plated onto YPDA or SD-ura medium solidified with 1.5% (w/v) Bacto-agar. Plates were incubated for 3 to 4 days at 30°C before counting the number of colony-forming units. Relative survival values were calculated by dividing the number of colonies obtained after 24 hours 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 done at least in triplicate. Results are presented as mean ± SE.

**Determination of Drug Sensitivity with Plate Assays**

To determine drug sensitivity using plates containing inhibitors, cultures were grown to an A₆₀₀ = 0.3. Serial 1:10 dilutions in water were prepared, and 3-μL aliquots of each dilution were spotted onto drug-containing plates. The plates were incubated 2 to 3 days at 30°C and then photographed.

**Drugs**

Etoposide and mAMSA were obtained from Sigma (Atlanta, GA) and Bristol-Myers Squibb (New York, NY), respectively. Drugs were prepared as 20 mg/mL stock solutions in 100% DMSO and stored in small aliquots at −20°C.

**Results**

**Repair-Proficient *S. cerevisiae* Strains Expressing Wild-type and Drug-Hypersensitive Allele of Topoisomerase II Enzyme Show Enhanced Sensitivity to Topoisomerase II – Targeting Drugs**

Repair-proficient yeast cells have limited sensitivity to most common agents that target topoisomerase II. We showed previously that overexpression of yeast topoisomerase II in rad52Δ cells greatly enhanced sensitivity to a wide range of topoisomerase II–targeting agents, including etoposide and mAMSA (30). We also showed that over-expression of drug-hypersensitive alleles increases the sensitivity beyond that seen with similar levels of expression of the wild-type enzyme in rad52Δ-deficient cells (36). These results suggested that overexpression of either wild-type topoisomerase II or a drug-hypersensitive variant could be used to determine whether specific genes play roles in cell survival following exposure to topoisomerase II–targeting agents. To explore this strategy, we first examined the level of drug sensitivity seen when wild-type or drug-hypersensitive alleles of TOP2 were overexpressed in cells that were completely repair proficient. We hypothesized that in order for a strain to be useful for assessing the role of genes that may confer sensitivity to topoisomerase II–targeting agents we needed to show cell killing in a repair-proficient strain under defined conditions. As in previous studies, we chose etoposide and mAMSA as model topoisomerase II poisons (30, 34, 37). Etoposide was chosen because it is in common clinical use and because it is an example of a topoisomerase II–targeting drug that does not intercalate in DNA but instead binds directly to the enzyme (38, 39). Many different intercalating agents target topoisomerase II (40); mAMSA is a potent intercalating topoisomerase II–targeting drug that kills yeast cells primarily due to its action against TOP2 (34, 41).

Overexpression of wild-type topoisomerase II enzyme in the repair-proficient strain JN362a resulted in increased sensitivity to etoposide compared with cells carrying an empty vector (yCP50; Fig. 1A). Etoposide concentrations below 100 μg/mL resulted in greater growth inhibition than seen with the empty vector, whereas concentrations at ≥100 μg/mL resulted in cell killing (survival dropping below 100%) after 24-hour etoposide exposure. Cells expressing the Ser²⁴⁶Trp allele showed cell killing at much lower etoposide concentrations. Relative survival after 24-hour exposure to 20 μg/mL etoposide was <10%, although higher etoposide concentrations resulted in little additional cell killing.

Figure 1B shows a similar set of experiments assessing the sensitivity of cells to the intercalating topoisomerase II poison mAMSA. Overexpression of wild-type topoisomerase II enhances the growth inhibition of cells by mAMSA, although cell killing was not seen at the highest concentration tested (100 μg/mL). The overexpression of the mAMSA-hypersensitive allele of topoisomerase II (Thr²⁴⁴Pro) resulted in a further increase in sensitivity, with cell killing occurring following exposure to mAMSA concentrations of ≥50 μg/mL (Fig. 1B). Taken together, these results show that overexpression of either wild-type or drug-hypersensitive alleles of TOP2 confer substantial sensitivity to topoisomerase II–targeting drugs and that the level of sensitivity observed is likely to be sufficient to allow examination of the effects of mutations that may further enhance sensitivity of cells to these agents. It should be noted that the Ser²⁴⁶Trp allele does not change sensitivity to mAMSA, nor does the Thr²⁴⁴Pro allele change sensitivity to etoposide (refs. 35, 36; data not shown). Therefore, to accurately examine the effects of etoposide and mAMSA, two different plasmids carrying the two alleles needed to be used.
Sensitivity of yku70 and yku80 Mutant Cells to Topoisomerase II–Targeting Drugs

We next examined the effect of topoisomerase II–targeting drugs on isogenic strains carrying mutations in the yku70 or yku80 genes. Both yku70 and yku80 are required for NHEJ (27, 42) and both mutants show equivalent defects in both nonhomologous recombination and sensitivity to DNA-damaging agents. Figure 2A shows the sensitivity to etoposide of strains defective in Δyku80 compared with wild-type cells. Data from Fig. 1 for the wild-type strain JN362a are also shown to compare the sensitivity of Δyku80 and wild-type cells. Neither wild-type cells nor Δyku80 cells showed significant etoposide sensitivity when the cells carried an empty vector (yCP50). Δyku80 cells overexpressing wild-type TOP2 were somewhat more sensitive to etoposide exposure than wild-type cells. When we compared the survival at 200 μg/mL etoposide using Student’s t test, the difference was statistically significant (P = 0.02), although survival differences at etoposide concentrations below 50 μg/mL were not statistically significant. The effect of the Δyku80 mutation on cells overexpressing the Ser740Trp allele of yeast topoisomerase II showed a greater effect on

Figure 1. Sensitivity of repair-proficient strains overexpressing TOP2 alleles to etoposide and mAMSA. Cytotoxicity of topoisomerase II–targeting drugs was assessed in a repair-proficient S. cerevisiae strain JN362a transformed with yCP50 (empty vector), pDED1Top2 (overexpressing wild-type topoisomerase II), and pDED1yTOP2S740W and pDED1yTOP2T744P (expressing drug-hypersensitive topoisomerase II alleles Ser740Trp and Thr744Pro, respectively). Logarithmically growing cultures were diluted to 2 × 10^6 cells/mL and the cells were exposed for 24 h to the indicated concentrations of etoposide (A) and mAMSA (B). Aliquots were removed and diluted samples were plated on synthetic medium lacking uracil to determine cell viability. Plates were incubated for 3 to 4 d at 30 °C before the numbers of colonies were counted. Survival rates are expressed in percentages relative to the number of viable colonies at the time of drug addition. Points, mean of three independent experiments; bars, SE. Results with wild-type strains shown in Figs. 2 to 6 replot the data shown in this figure.

Sensitivity of yku70 and yku80 Mutant Cells to Topoisomerase II–Targeting Drugs

We next examined the effect of topoisomerase II–targeting drugs on isogenic strains carrying mutations in the yku70 or yku80 genes. Both yku70 and yku80 are required for NHEJ (27, 42) and both mutants show equivalent defects in both nonhomologous recombination and sensitivity to DNA-damaging agents. Figure 2A shows the sensitivity to etoposide of strains defective in Δyku80 compared with wild-type cells. Data from Fig. 1 for the wild-type strain JN362a are also shown to compare the sensitivity of Δyku80 and wild-type cells. Neither wild-type cells nor Δyku80 cells showed significant etoposide sensitivity when the cells carried an empty vector (yCP50). Δyku80 cells overexpressing wild-type TOP2 were somewhat more sensitive to etoposide exposure than wild-type cells. When we compared the survival at 200 μg/mL etoposide using Student’s t test, the difference was statistically significant (P = 0.02), although survival differences at etoposide concentrations below 50 μg/mL were not statistically significant. The effect of the Δyku80 mutation on cells overexpressing the Ser740Trp allele of yeast topoisomerase II showed a greater effect on

Figure 2. Sensitivity of Δyku80 strains overexpressing topoisomerase II alleles to etoposide and mAMSA. Sensitivity of Δyku80 cells transformed with plasmids expressing either wild-type or drug-hypersensitive topoisomerase II enzymes to etoposide (A) and mAMSA (B). Data for the repair-proficient strain JN362a from Fig. 1 are also plotted to illustrate the differences observed between the mutant and repair-proficient strains. *, P < 0.05, significant differences from the corresponding wild-type samples (Student’s t test).
etoposide sensitivity. Concentrations of etoposide greater than 50 μg/mL resulted in significantly increased cell killing compared with the wild-type strain expressing the Ser240Trp TOP2 allele. At 200 μg/mL etoposide, relative survival was ~0.2% for the yku80 strain compared with ~8% for the wild-type strain.

A somewhat different result was obtained when the sensitivity to mAMSA was examined in yku80 cells. The results of cell survival following exposure to various concentrations of mAMSA for 24 hours are shown in Fig. 2B. As was observed with etoposide, yku80 cells carrying yCP50 showed no sensitivity to mAMSA under the conditions of the experiment. There was also no significant difference in sensitivity to mAMSA between the wild-type strain and the yku80 strain when wild-type yeast topoisomerase II was overexpressed. However, yku80 cells overexpressing the Thr744Pro allele showed a significant increase in the cellular sensitivity at the highest mAMSA concentration tested (100 μg mAMSA/mL) compared with the wild-type strain. These results clearly indicate enhanced sensitivity to etoposide in cells defective in yku80 function, with a more equivocal effect for sensitivity of yku80 mutants to mAMSA.

Because yku70p and yku80p form a heterodimer, and mutations in either protein result in the same phenotype, we anticipated that we would observe similar effects on etoposide and mAMSA sensitivity in yku70 cells as was seen in yku80 cells. Results obtained with yku70 cells are shown in Fig. 3. The overall sensitivity to etoposide seen with yku80 cells was very similar to that seen in yku80 cells. However, the overexpression of wild-type TOP2 in yku70 cells did not result in a significant sensitization to etoposide compared with wild-type cells. The comparison of wild-type with yku80 cells at 200 μg/mL etoposide using Student’s t test gave a P = 0.08. Although this difference is not significant, it is very similar to the difference seen between wild-type and yku80 cells. As was seen with yku80-deficient cells, yku70 cells overexpressing Ser240Trp were significantly more sensitive to etoposide than wild-type cells overexpressing the etoposide-hypersensitive enzyme. In experiments examining mAMSA sensitivity (Fig. 3B), no significant difference in sensitivity was seen between wild-type and yku70 cells under any of the conditions tested. Taken together, the results support a role for both yku70 and YKU80 in sensitivity to etoposide, whereas effects of loss-of-function of these genes on mAMSA sensitivity was less clear.

Hypersensitivity of dnl4, lif1, and nej1 Mutants to Topoisomerase II Poisons

In addition to their roles in NHEJ, YKU70 and YKU80 have also been shown to play critical roles in the maintenance of telomere function and adaptation to DNA damage (11, 43–48). To examine whether the sensitivity conferred by yku70 and yku80 arose from a defect in NHEJ, we assessed the sensitivity of strains carrying mutations in other genes that function in this pathway. In all eukaryotes, NHEJ is completed by a specialized DNA ligase, DNA ligase IV. In yeast, DNA ligase IV is encoded by a single nonessential gene DNL4. Yeast also encodes an accessory protein that physically interacts with DNL4 termed LIF1 (49, 50), which is homologous to the mammalian ligase IV accessory protein XRCC4 (12, 20). We constructed strains carrying a deletion of LIF1 and examined their sensitivity to etoposide and mAMSA. The results obtained with Δlif1 strains are shown in Fig. 4. As was observed with the other NHEJ strains, deletion of LIF1 does not result in sensitivity to etoposide (Fig. 4A) or mAMSA (Fig. 4B) in cells carrying yCP50, nor was any increase in sensitivity seen for either drug in Δlif1 cells overexpressing wild-type yeast TOP2. Notably, even the small increase in sensitivity seen with etoposide in cells...
overexpressing wild-type TOP2 in the yku70 or yku80 mutant strains was not observed. Nonetheless, a significant increase in the cell killing with etoposide was observed for cells expressing the Ser740Trp allele of topoisomerase II enzyme at several different etoposide concentrations. As shown in Fig. 4B, Dlif1 cells also showed enhanced sensitivity to mAMSA in cells expressing the mAMSA-hypersensitive allele Thr744Pro. The enhanced cell killing in Dlif1 cells was observed at several mAMSA concentrations ranging from 20 to 100 μg mAMSA/mL.

Figure 4. Sensitivity of Dlif1 strains overexpressing either wild-type or drug-hypersensitive alleles of yeast topoisomerase II to topoisomerase II poisons. Sensitivity of Dlif1 expressing wild-type and Ser740Trp allele of topoisomerase II to etoposide (A). Sensitivity to Dlif1 expressing wild-type and Thr744Pro mutant allele of topoisomerase II to mAMSA (B). The cells were exposed to the indicated drug concentrations in liquid culture as described in Materials and Methods and plated to determine viable titers. Survival rates are expressed in percentages relative to the number of viable colonies at the time of drug addition. The data for the repair-proficient strain JN362a (WT) are also plotted to illustrate the differences observed between the mutant and repair-proficient strains. *, P < 0.05, significant differences from the corresponding wild-type samples (Student’s t test).

Figure 5. Sensitivity to mAMSA and etoposide in Ddnl4 deletion strain overexpressing either wild-type or drug-hypersensitive alleles of yeast topoisomerase II. Sensitivity of Ddnl4 expressing wild-type and Ser740Trp allele of topoisomerase II to etoposide (A). Sensitivity to Ddnl4 expressing wild-type and Thr744Pro mutant allele of topoisomerase II to mAMSA (B). The cells were exposed to the indicated drug concentrations in liquid culture as described in Materials and Methods and plated to determine viable titers. Survival rates are expressed in percentages relative to the number of viable colonies at the time of drug addition. The data for the repair-proficient strain JN362a (WT) are also plotted to illustrate the differences observed between the mutant and repair-proficient strains. *, P < 0.05, significant differences from the corresponding wild-type samples (Student’s t test).

Strains lacking DNL4 exhibited etoposide sensitivity similar to that seen with Dlif1 strains (Fig. 5A). No increase in etoposide sensitivity was seen in cells overexpressing wild-type TOP2. Enhanced etoposide sensitivity was observed when cells expressed the etoposide-hypersensitive TOP2 allele Ser740Trp. Whereas the sensitivity at high concentrations of etoposide was similar between the Ddnl4 and Dlif1 strains, at low etoposide concentrations (e.g., 20 μg/mL etoposide), the Dlif1 strain was clearly more sensitive than the Ddnl4 strain (compare Fig. 4A with
Fig. 5A). Unlike our observation with Δlif1 strains, Δdnl4 cells did not exhibit hypersensitivity to mAMSA even when expressing the mAMSA-hypersensitive allele Thr241Pro. Therefore, Δdnl4 and Δlif1 strains show subtle differences in their sensitivities to both etoposide and mAMSA.

An additional gene that is required for NHEJ in yeast, NEJ1, functions as part of a complex with LIF1 and DNL4 (51, 52). NEJ1 also functions as a regulator of NHEJ in a cell type–specific manner (10, 52). Δnej1 cells did not exhibit enhanced sensitivity to etoposide or mAMSA when the cells carried yCp50 or overexpressed wild-type topoisomerase II, but Δnej1 cells exhibited enhanced sensitivity to both etoposide and mAMSA when the drug-hypersensitive alleles of topoisomerase II enzyme were expressed (Fig. 6). Therefore, Δnej1 strains exhibited the same pattern of hypersensitivity to etoposide and mAMSA seen with Δlif1 strains. Taken together, all three strains that have specific defects in NHEJ show hypersensitivity to etoposide and, again, somewhat equivocal effects with cells treated with mAMSA.

**Deletion of Other Repair Genes in NHEJ-Defective Strains Enhances Hypersensitivity to Topoisomerase II Poisons**

All the experiments in the previous sections relied on topoisomerase II overexpression to show a role for NHEJ in repairing topoisomerase II–mediated DNA damage. To examine the importance of NHEJ in the context of normal levels of topoisomerase II expression, we used the same approach that had been applied previously to test the importance of NHEJ in the repair of damage due to ionizing radiation. We combined mutations that confer defects in NHEJ with a deletion of the RAD52 gene, thereby eliminating homologous recombination as a pathway for repairing damage. We constructed strains carrying mutations in both pathways and examined sensitivity to either etoposide or mAMSA. In these experiments, the strains carried no plasmid, and sensitivity was examined in rich medium. The results obtained with Arad52 single-mutant strain and Aku70.Arad52 or Δnej1.Arad52 double-mutant strains are shown in Fig. 7. As we have shown previously, Arad52 single mutants are very sensitive to either etoposide or mAMSA. In these experiments, the strains exhibited hypersensitivity to both etoposide and mAMSA when the drug concentrations were >20 μg/mL or mAMSA concentration >7 μg/mL were cytotoxic when drug exposure was for 24 hours. Aku70 Arad52 mutants show cell killing with either drug at significantly lower concentrations, <10 μg/mL etoposide or ~3 μg/mL mAMSA. At higher drug concentrations, cell survival is significantly less in Aku70.Arad52 mutants than in Arad52 single mutants. Similar results are also seen with Δnej1.Arad52 double mutants, although the effect of Δnej1 seems to be less than the effect of Aku70 with both etoposide (Fig. 7A) and mAMSA (Fig. 7B). However, the difference between Aku70.Arad52 and Δnej1.Arad52 was not statistically significant. Nonetheless, these results clearly show that NHEJ participates in repair of topoisomerase II–mediated DNA damage in cells lacking homologous recombination.

We also examined the effect of combining deletions of other genes that may participate in the repair of DSBs with deletions in genes important for NHEJ. A gene that may play critical roles in both homologous and nonhomologous recombination pathways is the RAD1 gene. RAD1 is required for the single-strand annealing homologous recombination pathway (53) also participates in a yku70-independent NHEJ pathway (54, 55). For simplicity, we examined sensitivity of Arad1 and Arad1.Aku70 strains by spotting dilutions of logarithmically growing cultures onto plates containing either etoposide or mAMSA (Fig. 7C). Strains

**Figure 6.** Sensitivity to topoisomerase II poisons in Δnej1 deletion strain expressing either wild-type or drug-hypersensitive alleles of yeast topoisomerase II. Etoposide sensitivity of Δnej1 expressing wild-type and Ser740Trp mutant allele of topoisomerase II (A). mAMSA sensitivity to Δnej1 expressing drug-hypersensitive allele Thr744Pro mutant allele of topoisomerase II (B). The cells were exposed to the indicated drug concentrations in liquid culture as described in Materials and Methods and plated to determine viable titers. Survival rates are expressed in percentages relative to the number of viable colonies at the time of drug addition. The data for the repair-proficient strain JN362a (WT) are also plotted to illustrate the differences observed between the mutant and repair-proficient strains. P < 0.05, significant differences from the corresponding wild-type samples (Student’s t test).
carrying a deletion of RAD1 have similar sensitivities to both etoposide and mAMSA. Similar to the experiments shown in Fig. 7A and B, the cells express normal levels of wild-type topoisomerase II. A slight increase in sensitivity to both mAMSA and etoposide is seen in yku70 single mutants, but a much greater sensitivity to both drugs is seen in D rad1 D yku70 double mutants. These results further show that loss of other strand break repair pathways increases the requirement for the yku70-dependent NHEJ in the repair of topoisomerase II–mediated DNA damage.

Discussion

Etoposide and mAMSA target topoisomerase II by trapping an intermediate of the enzyme reaction cycle and are termed topoisomerase II poisons. Processing of topoisomerase-DNA covalent complexes can generate DNA DSBs. Treatment of mammalian cells with etoposide or other topoisomerase II poisons leads rapidly to the generation of DNA DSBs as shown by the appearance of Mre11/Rad50/Nbs1 and Rad51 foci (56, 57), and more direct assays of strand breaks using neutral comet assays (58, 59). The DSBs generated by topoisomerase II poisons are likely to be the major determinant of cell killing by these drugs. In this work, we have shown that mutations in different components of the yeast NHEJ pathway lead to hypersensitivity to drugs targeting topoisomerase II. We were able to observe significant sensitivity to topoisomerase II–targeting drugs even in cells that were only deficient in NHEJ, provided that we increased the sensitivity by overexpression of the drug target and used mutant alleles that generate higher levels of DNA damage at low drug concentrations.

Previous studies in yeast have shown the importance of DSB repair pathways in cell survival following exposure to topoisomerase II poisons. Mutations in genes required for homologous recombination, such as RAD52, result in substantially increased sensitivity to etoposide and mAMSA (5, 60). Subsequent work has shown that mutations in other genes in the RAD52 epistasis group also confer hypersensitivity to topoisomerase II poisons. These studies clearly showed that DSB repair was important for cell survival following exposure to topoisomerase II poisons and that in yeast the major DSB repair pathway used is homologous recombination. Mutations in RAD50 and MRE11 result in defects in both homologous recombination and NHEJ (9, 13). Cells defective in these genes are also strongly hypersensitive to topoisomerase II poisons, although it is unclear whether the hypersensitivity was due solely to defects in homologous recombination or whether defects in nonhomologous recombination also contribute to overall sensitivity in mutants defective in mre11 or rad50.

Topoisomerase II–targeting drugs lead to deletions and translocations in mammalian cells, which may be mediated...
by drug-induced DSBs. These genome rearrangements are an important aspect of clinical treatment with topoisomerase II–targeting drugs, especially etoposide, because etoposide treatment can lead to secondary malignancies. The most common oncogenic translocation that has been seen in etoposide-treated patients involves the MIH1 gene as one of the two translocation partners (28, 61, 62). Translocations involving the MIH1 gene can result in myeloid leukemia with very poor prognosis (29, 63). The mechanisms of drug-induced translocations are poorly understood. Recently, the induction of homologous and nonhomologous recombination in response to etoposide treatment was examined using a yeast assay, and nonhomologous recombination events were detected (7). A plausible hypothesis is that the nonhomologous recombination events arise from processing topoisomerase II–mediated damage by the NHEJ pathway. These observations suggest that yeast may be a model system for understanding the mechanistic details of drug induced chromosome alterations.

Because the clearest effects of etoposide were seen with the drug-hypersensitive allele of TOP2, it was possible that part of the effect arose from the unique properties of the Ser740Trp allele. We do not favor this possibility because combining deletions in genes required for end-joining and deletions in RAD52 or RAD1 resulted in enhanced sensitivity compared with either single mutant. Nonetheless, the protein encoded by topoisomerase II (Ser740Trp) has biochemical properties in the presence of etoposide that are distinct from the wild-type protein. In addition to elevated DNA cleavage in the presence of etoposide, the covalent complexes formed in vitro with topoisomerase II (Ser740Trp) in the presence of etoposide are more heat and salt stable than those formed with the wild-type protein. It is possible that some covalent complexes formed by this protein may be essentially irreversible. Irreversible topoisomerase II-DNA covalent complexes may pose additional challenges for repair that specifically require NHEJ. Although the mutation Ser740Trp accentuates the heat stability of topoisomerase II-DNA covalent complexes formed in the presence of drug, some covalent complexes formed by wild-type topoisomerase II in the presence of etoposide have enhanced heat and salt stability compared with those formed in the presence of intercalating drugs, such as mAMSA (64, 65). Differences in the stability of covalent complexes may contribute to the differential effect we observed between mAMSA and etoposide. It is also interesting to note that whereas etoposide is a very effective drug the clinical activity of mAMSA has been disappointing.

Although this difference in clinical activity may be due to other aspects of the drug, the ability to generate a specific class of lesions may contribute both to the clinical efficacy of etoposide and to its ability to generate translocations. Because topoisomerase II–targeting agents, such as clorocidin, can generate high levels of irreversible topoisomerase II-DNA covalent complexes (66), it will be interesting to determine whether these irreversible complexes must be repaired by NHEJ pathways.

The experimental strategy of using overexpression of topoisomerase II and the use of drug-hypersensitive alleles is an effective approach for examining the repair of lesions induced by the enzyme. We are able to effect cell killing with either etoposide or mAMSA in otherwise repair-proficient cells. We are currently applying this strategy to identify other pathways important for repairing topoisomerase II–mediated DNA damage.

References

Nonhomologous End-Joining Pathways and Etoposide


Molecular Cancer Therapeutics

Roles of nonhomologous end-joining pathways in surviving topoisomerase II–mediated DNA damage


Updated version  
Access the most recent version of this article at:  
http://mct.aacrjournals.org/content/5/6/1405

Cited articles  
This article cites 65 articles, 35 of which you can access for free at:  
http://mct.aacrjournals.org/content/5/6/1405.full#ref-list-1

Citing articles  
This article has been cited by 10 HighWire-hosted articles. Access the articles at:  
http://mct.aacrjournals.org/content/5/6/1405.full#related-urls

E-mail alerts  
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.