

# DNA Damage-Processing Pathways Involved in the Eukaryotic Cellular Response to Anticancer DNA Cross-Linking Drugs

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## ABSTRACT

We used a panel of isogenic *Saccharomyces cerevisiae* strains compromised in several different DNA damage-processing pathways to assess in vivo processing of DNA adducts induced by four cross-linking anticancer drugs. By examining cytotoxicity profiles, cell cycle arrest patterns, and determining recombination and mutation frequencies, we found that cisplatin-, nitrogen mustard-, mitomycin-, and carmustine-induced DNA adducts in *S. cerevisiae* are processed by components of the nucleotide excision repair (NER), recombination repair (RR), and translesion synthesis (TLS) pathways, with substantially different contributions of each pathway for the drugs studied here. In contrast to previous studies that used single pathway-compromised strains to identify genes that mediate sensitivity to DNA cross-linking drugs, we used strains that were compromised in multiple pathways. By doing so, we were able to

establish several functions that were previously unknown and interconnections between different DNA damage-processing pathways. To our surprise, we found that for cisplatin-induced cytotoxicity, TLS and RR contribute to survival to a significant extent. In the case of nitrogen mustard DNA adduct processing, equal involvement of two major pathways was established: one that requires functional RR and NER components and one that requires functional TLS and NER components. These data reveal the complexity of DNA cross-link processing that, in many cases, requires interactions of components from several different DNA damage-processing systems. We demonstrate the usefulness of yeast strains with multiple simultaneous defects in DNA damage-processing pathways for studying the modes of action of anticancer drugs.

Compounds that form chemical bonds with DNA molecules represent several important classes of anticancer drugs. A large number of such compounds have been synthesized and tested for anticancer activity, and many of them are in clinical use. These drugs include nitrogen mustards, the aziridines, the alkane sulfonates, the nitrosoureas, and platinum compounds (Pratt, 1994; Lawley and Phillips, 1996; Tannock and Goldenberg, 1998). All of these drugs interact with a variety of biomolecules in cells, but the most important aspect responsible for their cytotoxicity seems to be inhibition of cell division or stimulation of apoptosis caused by the formation of DNA adducts (Tannock and Goldenberg, 1998). Important examples of such drugs include cisplatin, nitrogen mustard, mitomycin, and carmustine (Fig. 1) (Dronkert and Kanaar, 2001).

Once DNA adducts are formed, to carry out normal DNA transactions, such as replication and transcription, cells must be able to remove or to tolerate the presence of such DNA

damage. Generally, base excision repair (BER) removes base damage that causes relatively minor distortions in DNA (Memisoglu and Samson, 2000). Nucleotide excision repair (NER) is a major pathway by which cells remove bulky, DNA helix-distorting adducts (Friedberg et al., 1995). Mismatch repair (MMR) corrects errors (insertions, deletions, and/or base substitutions) created during DNA replication (Harfe and Jinks-Robertson, 2000). Homologous recombination repair (RR) and translesion synthesis (TLS) also provide routes by which cells can continue replication despite the presence of replication fork-blocking adducts (Doetsch et al., 2001). RR and TLS are often regarded as DNA damage-tolerance pathways because they allow cells to complete replication and mitosis at the expense of increasing mutation and recombination frequencies. These pathways are summarized in Fig. 2.

Resistance of cancer cells to chemotherapeutic drugs is a major problem encountered in the treatment of tumors (Tannock and Goldenberg, 1998). The basis for resistance has been studied extensively and can be drug-specific, or nonspecific as in the case of alkylating and platinum drugs (Pratt,


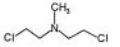
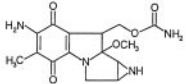
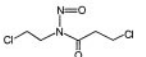
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**ABBREVIATIONS:** BER, base excision repair; NER, nucleotide excision repair; MMR, mismatch repair; RR, recombination repair; TLS, translesion synthesis; YEPD, medium composed of 1% yeast extract, 2% Bacto-peptone, 2% dextrose, 2% agar, and 0.005% adenine sulfate.

1994; Tannock and Goldenberg, 1998). Increased removal and/or replicative bypass of DNA adducts have been found to be among the major mechanisms by which cancer cells reverse the effects of intra- and interstrand cross-linking drugs (Kartalou and Essigmann, 2001; Panasci et al., 2002). Despite the existence of a large body of data characterizing DNA cross-link repair, the specific components of DNA damage-processing pathways and their individual contributions to the repair of cross-links are poorly understood. Exploring the influence of these pathways on cell survival and defining their potential relationships with each other are crucial for understanding the modes of action of DNA cross-linking anticancer drugs used currently in the clinic.

In this work, we used a variety of isogenic *Saccharomyces cerevisiae* strains compromised in one or two DNA damage-processing pathways to identify the components that are crucial for cell survival after exposure to cisplatin, nitrogen mustard, mitomycin, and carmustine (Fig. 1). A second objective was to determine potential interrelationships between these DNA damage-processing pathways. *S. cerevisiae* is an attractive model system for such studies because of the availability of multiple, isogenic mutants, short generation times, and the similarity of yeast DNA damage-processing pathways to those of mammalian systems (Friedberg et al., 1995; Perego et al., 2000; Prakash and Prakash, 2000).

For these studies, we used strains compromised in one or two of the following pathways: NER, RR, TLS, MMR, and BER (Fig. 2). These pathways are crucial for DNA repair during replication, and postreplication repair is carried out mainly by TLS or RR (Sonoda et al., 2003). Previous studies have examined the repair of DNA lesions induced by cisplatin and nitrogen mustard in *S. cerevisiae* using nonisogenic, single-pathway defective strains (Hannan et al., 1984; Durrant et al., 1999; Grossmann et al., 1999, 2000; McHugh et al., 2000). Our panel of isogenic strains allows for assessment of the contributions of each pathway in DNA cross-link processing. These multiple pathway-compromised strains also reveal potential overlap or competition among pathways (Swanson et al., 1999). In addition, studies aimed at identifying key determinants of drug-specific cell sensitivity could provide guidance for design of combination chemotherapy strategies for clinical trials (Ferguson, 1990).

Drug	Formula	Cytotoxic adduct	DNA distortion
Cisplatin		Intrastrand 5'-G*G*-3'	Major
Nitrogen mustard		Interstrand 5'-G*XC-3' 3'-CXG*-5'	Major
Mitomycin C		Interstrand 5'-CG*-3' 3'-G*C-5'	Minor
Carmustine		Interstrand 5'-G*-3' 3'-C*-5'	Unknown

**Fig. 1.** Structures of and major DNA lesions induced by four anticancer cross-linking agents used in this study. DNA bases involved in a chemical cross-link with a specific drug are indicated by \* together with the extent of distortion caused by each of the four agents.

## Materials and Methods

**Yeast Strains.** Genotypes of strains used in this study are listed in Table 1. All strains used containing defects in NER, RR, BER, and TLS are isogenic derivatives of DSC0025 and were constructed as described previously (Swanson et al., 1999). Isogenic derivatives of SJR0769, the strains used to study contributions of MMR to cell sensitivity, were kindly provided by S. Jinks-Robertson (Emory University). Yeast strains were grown nonselectively on YEPD medium (1% yeast extract, 2% Bacto-peptone, 2% dextrose, 2% agar, and 0.005% adenine sulfate) (Swanson et al., 1999).

**Drugs.** Cisplatin, nitrogen mustard, carmustine, and mitomycin were purchased from Sigma-Aldrich (St. Louis, MO). Compounds were dissolved in water (cisplatin, nitrogen mustard, and mitomycin) or dimethyl sulfoxide (carmustine) and prepared as 10 mM (cisplatin, nitrogen mustard, and carmustine) or 2 mM (mitomycin) stock solutions and diluted appropriately to yield final concentrations (in water) between 10  $\mu$ M and 2.4 mM. Drugs were prepared as fresh solutions and used immediately for each experiment.

**Cytotoxicity Assays.** For each experiment, yeast cells from frozen stocks ( $-80^{\circ}\text{C}$ ) were grown for 2 days on YEPD plates at  $30^{\circ}\text{C}$ . YEPD (10 ml) was inoculated with a single colony and grown overnight at  $30^{\circ}\text{C}$ . Overnight culture (20–50  $\mu$ l) was used to inoculate 25 ml of fresh YEPD solution. Cells were grown until a density of  $A_{600} = 0.550$  to 0.600 was reached (early exponential growth phase), then pelleted and washed with deionized sterile water. Aliquots (1 ml) of cell suspension in water (approximately  $2.4 \times 10^7$  cells/ml) were exposed to various concentrations of drugs in Eppendorf tubes for 2 h at  $30^{\circ}\text{C}$  with shaking as described previously (McAulity and Lippard, 1996). After exposure to drugs, cells were washed twice and plated in duplicate in dilutions that generally yielded between 50 and 200 cells per plate. Unexposed cells were used as controls. Cells that survived exposure to drugs yielded visible colonies that were scored 48 h after exposure to drugs. Results are expressed as a percentage of cells that survived exposure versus nonexposed cells. Each experiment was performed at least three times with yeast strains freshly streaked from  $-80^{\circ}\text{C}$  stocks.

**Measurement of Induced Recombination and Mutation Frequencies.** Two-day-old yeast colonies were inoculated into 10 ml of YEPD liquid medium for 24 h, and this culture was used to inoculate 100 ml of fresh YEPD, which was grown overnight to a density of approximately  $2.4 \times 10^7$  cells/ml (early exponential growth phase). Cells were harvested by centrifugation, washed once with sterile water, and resuspended in sterile water. Cisplatin and nitrogen mustard solutions were added to a 20-ml cell suspension, and cells were exposed to drugs for 2 h at  $30^{\circ}\text{C}$ . Aliquots (100  $\mu$ l) of appropriate dilutions were plated in duplicate onto complete minimal media plates to determine viable cell numbers. The remaining cells were pelleted, resuspended in approximately 300  $\mu$ l of sterile water, and plated in triplicate on selective medium lacking lysine to select recombinants and onto canavanine-containing medium (60 mg/l) to identify mutation frequencies in the *CAN1* locus. Lys<sup>+</sup> colonies and Can<sup>r</sup> colonies were counted 2 days after plating. Each data point represents an average of at least three independent experiments.

**Cell Cycle Analysis.** Asynchronous cells were exposed to various concentrations of drugs for 2 h as described above. After exposure, cells were precipitated and resuspended in complete media, and their growth was allowed to continue for an additional 5 to 6 h. Aliquots of cells were removed at 0, 60, 120, 180, 240, and 300 min, washed from the growth media, resuspended in absolute ethanol, and left at  $4^{\circ}\text{C}$  overnight. The next day, cells were washed twice with deionized water, resuspended in 50 mM citrate buffer, pH 7.0, and treated with 8  $\mu$ l of RNase I (10 mg/ml) for 1 h at  $50^{\circ}\text{C}$ . Next, 25  $\mu$ l of proteinase K (10 mg/ml) was added, and incubation continued for 1 h. Cell suspensions were transferred to BD Falcon tubes (BD Biosciences, San Jose, CA) containing 1 ml of 16  $\mu$ g/ml propidium iodide in 50 mM citrate buffer, briefly sonicated, and fluorescence-activated cell sorting analysis of DNA

content was performed for 10,000 cells at low speed on a BD FACSCalibur instrument (BD Biosciences).

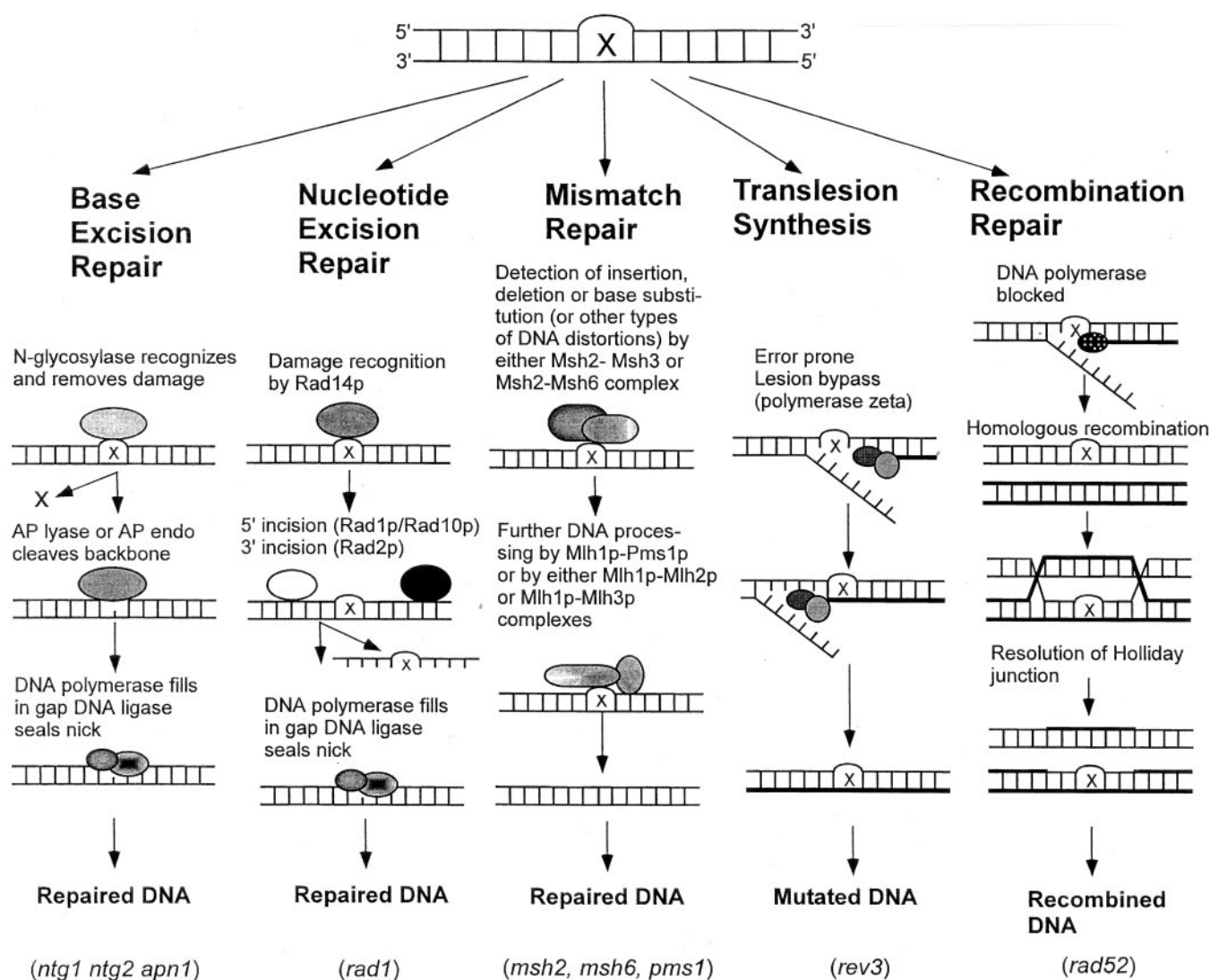
**Isobologram Analysis.** Data from the cytotoxicity assays were analyzed using CalcuSyn software (Biosoft, Ferguson, MO). The software analyzes drug interactions based on the method of Chou and Talalay (1984).

## Results

**Sensitivities of NER-, RR-, and TLS-Compromised Strains to Cisplatin and Nitrogen Mustard.** *S. cerevisiae* cells compromised in the NER, RR, and TLS pathways alone are sensitized to various extents to killing by DNA-damaging drugs (Hannan et al., 1984; Grossmann et al., 1999, 2000, 2001). To assess the sensitivities of DNA damage-processing pathway-compromised strains within the same genetic background, we exposed NER-, RR-, and TLS-compromised strains to various concentrations of cisplatin or nitrogen mustard and established that the TLS-compromised strain

was the least sensitive and the RR-compromised strain was the most sensitive to cisplatin exposure (Fig. 3A). In the case of nitrogen mustard (Fig. 3B), only the NER-compromised strain showed a moderate sensitivity compared with the wild-type strain, and the RR- and TLS-compromised strains were only slightly sensitive. These data indicate involvement of NER, RR, and TLS in the processing of toxic cisplatin-induced lesions as well as involvement of NER in the processing of toxic nitrogen mustard-induced lesions with the additional participation of either RR or TLS pathways. Moderate sensitivity of the NER-compromised strain to nitrogen mustard (Fig. 3B) supports the proposal that additional DNA damage-processing pathways contribute significantly to the cellular response to this drug (McHugh et al., 2000).

**Multiple Pathway-Compromised Strains Reveal New Repair Components for the Cellular Response to Cisplatin and Nitrogen Mustard.** To delineate further the contributions of each DNA damage-processing pathway,



**Fig. 2.** Major pathways for processing DNA damage in *S. cerevisiae*. X represents types of DNA damage caused by anticancer DNA cross-linking agents that are potentially processed by one or more of the indicated pathways. Major steps of each pathway are shown as are some of the key components of that pathway. Strains used in this study containing mutations in DNA repair components resulting in a defective DNA damage-processing system (or combination of two systems) are indicated at the bottom of each pathway. The indicated DNA repair pathways (BER, NER, and MMR) result in restoration of DNA in its original undamaged state, whereas TLS and RR result in genetic instability (increase in mutation and recombination frequencies) and are DNA damage "tolerance" pathways.



strains with two defective pathways (NER/RR-, NER/TLS-, and RR/TLS-compromised) were assessed for sensitivity to cisplatin or nitrogen mustard. Inactivation of the NER pathway together with the RR or TLS pathway resulted in an additive increase in sensitivity to cisplatin relative to the NER-, RR-, or TLS-compromised strains, and no cells survived at exposure concentrations higher than 50  $\mu$ M (Fig. 3A). The NER/RR-compromised strain (possessing only the TLS pathway) was as sensitive as the TLS/NER-compromised strain (possessing only the RR pathway). These data indicate that in the absence of NER, the RR and TLS pathways are involved in processing of toxic cisplatin lesions with similar efficiencies.

To investigate further the role of NER for the repair of cisplatin-DNA lesions in the absence of RR and TLS, a RR/TLS pathway-compromised strain was used. The inactivation of RR together with TLS resulted in extreme sensitivity to cisplatin, and little cell survival was detected even at exposures to low drug concentrations (25  $\mu$ M). A greater-than-additive increase in sensitivity caused by the loss of both the RR and TLS pathways was observed in this double pathway-compromised strain at 25  $\mu$ M. This extreme sensitivity was greater than those observed for the NER/RR ( $P < 0.01$ ,  $P$  = Student's  $t$  test) and NER/TLS ( $P < 0.03$ ) double pathway-compromised strains. This increase in sensitivity suggests that in the absence of these two damage-processing pathways, NER alone cannot compensate for the functions of the RR and TLS pathways in the processing of cisplatin lesions in yeast. This result is somewhat surprising because it has been generally assumed that toxic cisplatin-DNA adducts are handled primarily by NER (Jamieson and Lippard, 1999).

In the case of nitrogen mustard, simultaneous disruption of any two pathways (among NER, RR, and TLS) resulted in a greater-than-additive increase in sensitivity for all double pathway-compromised strains studied (Fig. 3B). No cell survival was detectable at drug concentrations greater than 50  $\mu$ M. The greater-than-additive increase in sensitivity that

was found for all double pathway-compromised strains suggests that the combination of NER and RR and/or NER and TLS pathways recognize and process similar toxic lesions and also suggests that interconnections may exist between these pathways in processing interstrand cross-links induced by nitrogen mustard. Our finding that RR- and TLS-compromised strains are only slightly more sensitive than wild type, together with the extreme sensitivity observed for any of the double pathway-compromised mutants to nitrogen mustard, indicates that the RR and TLS pathways possess a significant capacity for processing toxic nitrogen mustard-induced DNA adducts (Fig. 3B).

**BER and MMR Pathways Are Not Involved in Removal of Cisplatin- or Nitrogen Mustard-Induced DNA Adducts.** To examine the potential biological role of the yeast BER proteins Ntg1p, Ntg2p, and Apn1p in the processing of cisplatin- and nitrogen mustard-induced DNA lesions, strains lacking all three of these proteins were assessed for survival after exposures to either drug. No differences in sensitivity to cisplatin were observed for the BER-compromised strain compared with the wild-type cells (data not shown). Strains compromised in both BER and NER (*ntg1 ntg2 apn1 rad1*), BER and RR (*ntg1 ntg2 apn1 rad52*), or BER and TLS (*ntg1 ntg2 apn1 rev3*) were examined for sensitivity to cisplatin. No significant differences in sensitivity to cisplatin or nitrogen mustard were observed for the BER/NER-, BER/RR-, and BER/TLS-compromised strains compared with the single NER-, RR-, and TLS-compromised strains, respectively (data not shown). The absence of significant differences in sensitivity indicates that the BER pathway is not involved in the processing of toxic cisplatin- or nitrogen mustard-DNA adducts in *S. cerevisiae*.

No difference in sensitivity was observed between the wild-type and MMR-compromised (*msh2 msh6 pms1*) strains in response to cisplatin (Fig. 4A) and nitrogen mustard (Fig. 4B). When specific MMR components were inactivated together with NER (*rad1 msh6* or *rad1 pms1* strains), cells retained the same sensitivity as the *rad1* single-mutant

TABLE 1  
Strains used in this study and their genotypes

Strain	Genotype	Phenotype
DSC0025	<i>MATa ade2-101<sub>oc</sub> his3<math>\Delta</math>200 ura3<math>\Delta</math>Nco lys2<math>\Delta</math>Bgl leu2-R</i>	WT1
DSC0035	<i>DSC0025, ntg1<math>\Delta</math>::LEU2 ntg2<math>\Delta</math>::hisG apn1<math>\Delta</math>::HIS3</i>	BER-defective
DSC0036	<i>DSC0025, rad1<math>\Delta</math>::hisG</i>	NER-defective
DSC0041	<i>DSC0025, rad52<math>\Delta</math>::URA3</i>	RR-defective
DSC0044	<i>DSC0025, ntg1<math>\Delta</math>::LEU2 ntg2<math>\Delta</math>::hisG apn1<math>\Delta</math>::HIS3 rad52<math>\Delta</math>::URA3</i>	BER/ RR-defective
DSC0047	<i>DSC0025, ntg1<math>\Delta</math>::LEU2 ntg2<math>\Delta</math>::hisG apn1<math>\Delta</math>::HIS3 rev3<math>\Delta</math>::KAN<sup>R</sup></i>	BER/TLS-defective
DSC0051	<i>DSC0025, ntg1<math>\Delta</math>::LEU2 ntg2<math>\Delta</math>::hisG apn1<math>\Delta</math>::HIS3 rad1<math>\Delta</math>::hisG</i>	BER/NER-defective
DSC0051	<i>DSC0025, rad1<math>\Delta</math>::hisG rad52<math>\Delta</math>::URA3</i>	NER/ RR-defective
DSC0055	<i>DSC0025, rad1<math>\Delta</math>::hisG rev3<math>\Delta</math>::KAN<sup>R</sup></i>	NER/TLS-defective
DSC0118	<i>DSC0025, rev3<math>\Delta</math>::KAN<sup>R</sup> rad52<math>\Delta</math>::URA3</i>	TLS/RR-defective
DSC0133	<i>DSC0025, rev3<math>\Delta</math>::KAN<sup>R</sup></i>	TLS-defective
SJR0769	<i>MATa ade2-101oc his3<math>\Delta</math>200 ura3-Nhe lys2<math>\Delta</math>RV::hisG leu2-R(HIS3::intron::cb2 100% IR)-LEU2 Gal+</i>	WT2
SJR0755	<i>SJR0769, msh6::hisG</i>	MMR-defective
SJR0753	<i>SJR0769, msh2::hisG</i>	MMR-defective
SJR1183	<i>SJR0769, pms1::hisG</i>	MMR-defective
SJR1200	<i>SJR0769, rad1::hisG pms1::hisG-URA3-hisG</i>	NER-defective
SJR1231	<i>SJR0769, rad1::hisG msh6::hisG-URA3-hisG</i>	NER/MMR-defective
SJR1232	<i>MATa ade2-101<sub>oc</sub> his3<math>\Delta</math>200 ura3<math>\Delta</math>Nco-lys2<math>\Delta</math>3'-URA3 lys2<math>\Delta</math>Bgl leu2-R</i>	NER/MMR-defective
SJR897	<i>SJR897, rad1<math>\Delta</math>::hisG</i>	WT3
SJR925	<i>SJR897, rev3<math>\Delta</math>::KAN<sup>R</sup></i>	NER-defective
SJR943	<i>SJR897, rad1<math>\Delta</math>::hisG rev3<math>\Delta</math>::KAN<sup>R</sup></i>	TLS-defective
SJR1450		NER/TLS-defective

WT, wild type.

strain. These results indicate that the MMR pathway does not play a major role in the processing of either cisplatin- or nitrogen mustard-induced toxic adducts in *S. cerevisiae*.

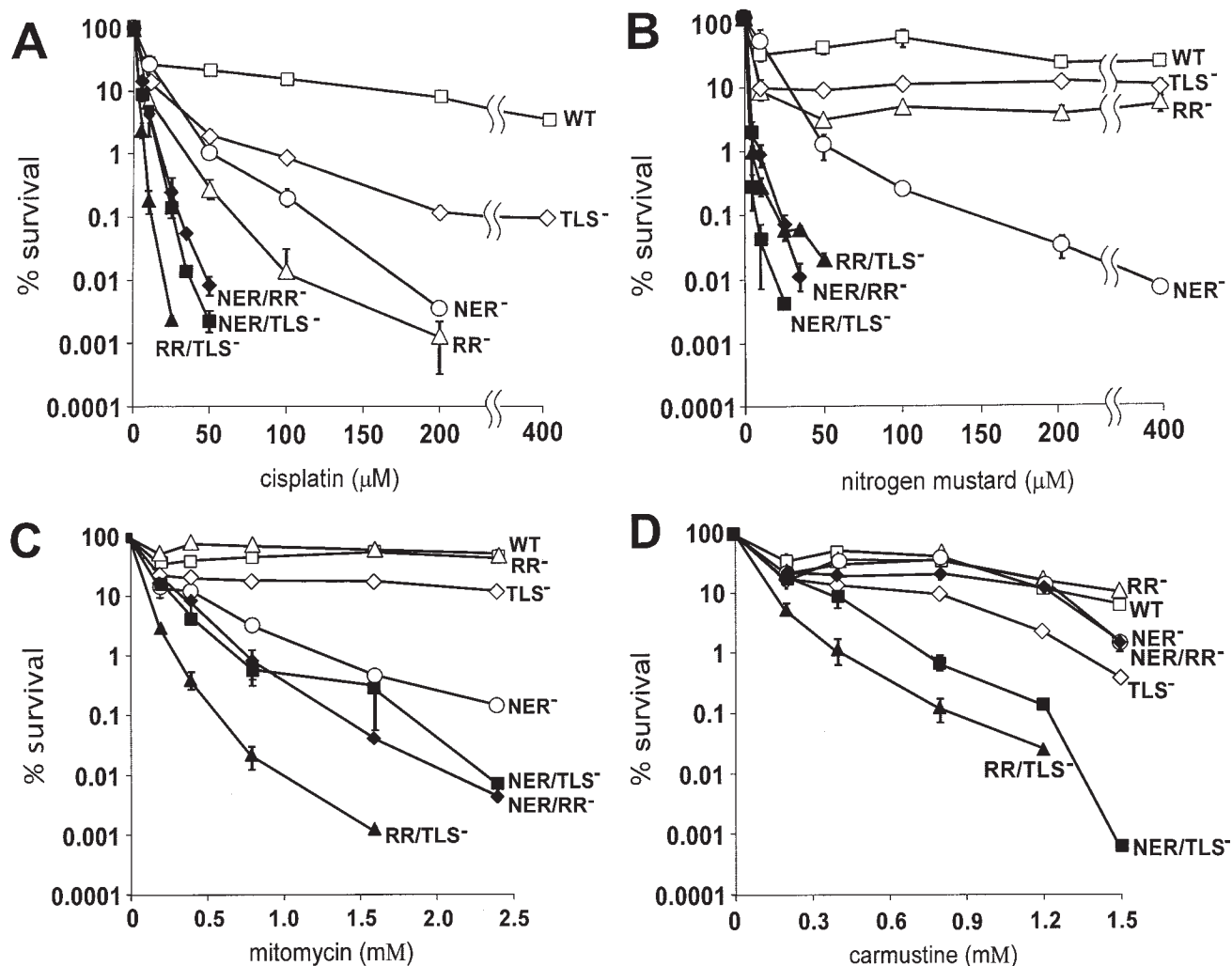
**Roles of NER, RR, and TLS Pathways in the Cellular Response to Mitomycin or Carmustine.** Mitomycin produces both DNA monoadducts and interstrand cross-links (Pratt, 1994; Tannock and Goldenberg, 1998). However, the yeast cellular toxicity profiles for this drug (Fig. 3C) are quite different from those observed for nitrogen mustard (Fig. 3B) or cisplatin (Fig. 3A). Relatively higher concentrations of mitomycin were necessary to produce toxicities equivalent to those achieved with nitrogen mustard and cisplatin. Double pathway-compromised strains (NER/RR, NER/TLS, and RR/TLS) exhibit less sensitivity to mitomycin compared with either nitrogen mustard or cisplatin. For the single pathway-compromised strains, the highest sensitivity to mitomycin was found for the NER-compromised strain, and for the double pathway-compromised strains, the highest sensitivity was found for the RR/TLS-compromised strain. These results indicate the importance of NER and TLS as well as RR for the processing of mitomycin-induced toxic lesions.

Relatively higher concentrations of carmustine were also required to achieve cytotoxic effects comparable with nitro-

gen mustard or cisplatin. All strains that were slightly-to-moderately sensitive to carmustine (TLS-, NER/TLS-, and RR/TLS-compromised strains) possess a common defect in TLS (Fig. 3D). This finding indicates the central importance of TLS and the participation of NER and RR in the processing of carmustine-induced toxic lesions.

The pronounced differences in the sensitivities to cisplatin, nitrogen mustard, mitomycin, and carmustine observed for the DNA damage-processing pathway-compromised strains indicate that the participation of a pathway and its capacity for handling cross-linking drug-induced toxic DNA damage are different among these four drugs. These results reveal the diverse nature of the processing pathways for the different types of drug-induced DNA cross-links.

**Combination Drug Experiments Reveal New Pathway Targets for Cytotoxicity.** Our results reveal that the processing of toxic drug-induced DNA cross-links requires participation of components from several pathways. The extent to which a particular pathway participates in DNA adduct processing seems to be drug-specific. This information can be used to design an effective cytotoxic combination of two or more drugs as well as to examine the genetic background in which this combination may yield the highest level



**Fig. 3.** Cisplatin (A), nitrogen mustard (B), mitomycin (C), and carmustine (D) sensitivities of DNA damage-processing pathway-defective yeast strains are shown. Cells were exposed for 2 h to various concentrations of drugs as described under *Materials and Methods*. Error bars represent the standard error for at least three experiments. Repair backgrounds of strains analyzed are indicated.

of cytotoxicity. Because two different pathways that share a common component may be involved in handling of drug-DNA lesions (Dronkert and Kanaar, 2001), exposure of cells to one drug may saturate the capacity of this repair component, effectively eliminating its participation in another pathway required for handling different DNA damage caused by the second drug. Simultaneous exposure to both drugs then results in greatly potentiating the cytotoxic effect of the second drug. To address this concept, we exposed several strains to either a combination of nitrogen mustard and carmustine or a combination of nitrogen mustard and cisplatin.

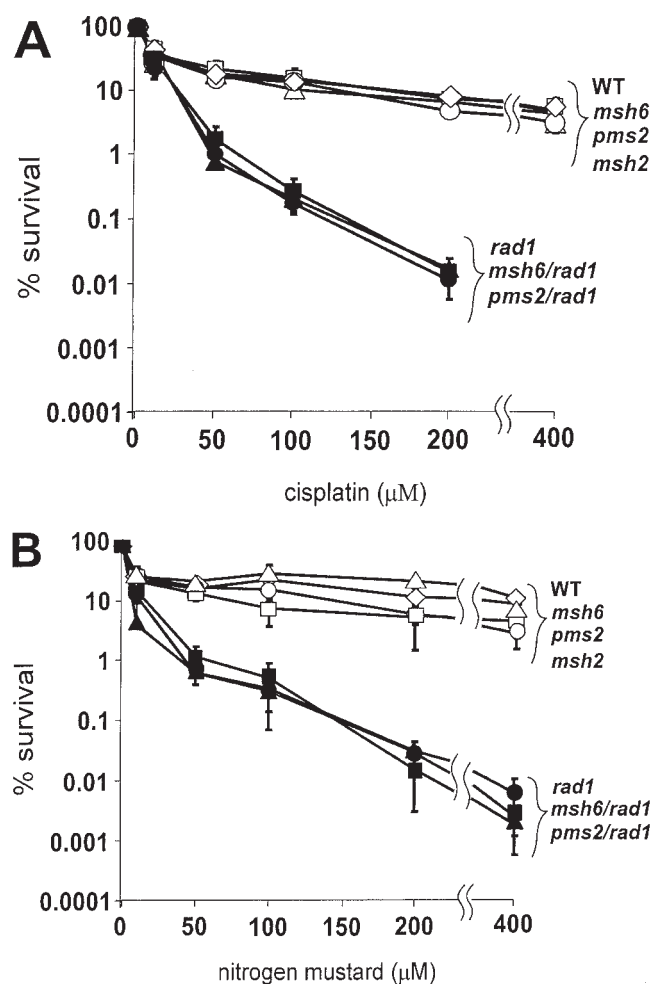
Although it has been reported previously that toxic nitrogen mustard adducts can be processed by using combined elements of either the TLS and NER or RR and NER pathways (Dronkert and Kanaar, 2001), toxic carmustine DNA adducts are processed primarily by TLS (Fig. 3D). In the case of cisplatin, toxic adducts are processed by the NER, RR, and TLS pathways (Fig. 3A). Cell toxicity data for these combination drug experiments are presented in Fig. 5. To determine the nature of interactions between nitrogen mustard and either carmustine or cisplatin, we conducted experiments in which the molar ratio of each drug combination was

varied and subjected to isobologram analysis (Berenbaum, 1978) (Fig. 6). The combination of nitrogen mustard and either carmustine or cisplatin results in synergistic toxicity for all strains tested for most ranges of molar ratios.

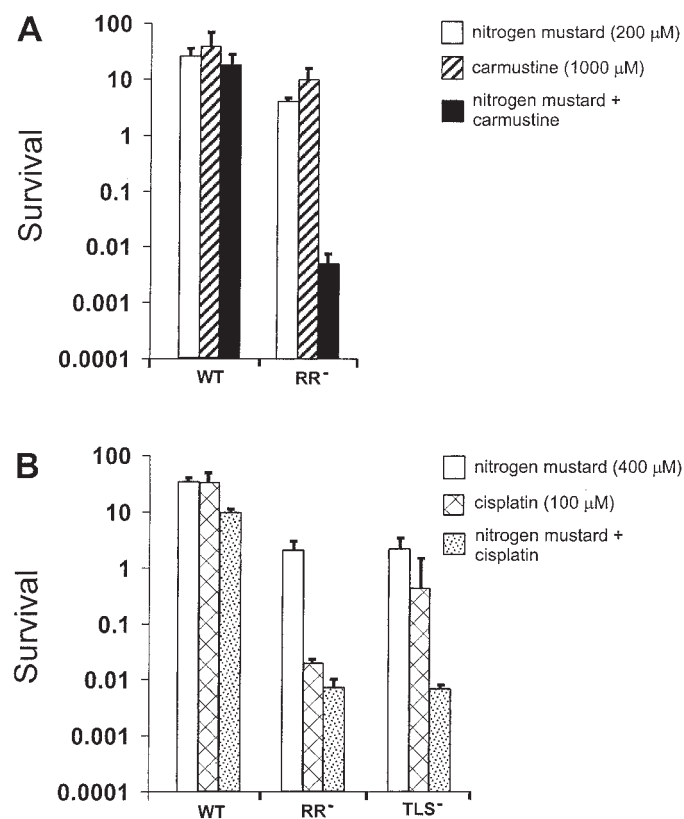
In the wild-type strain, compared with single drug exposures, only a modest additional increase in sensitivity was observed upon simultaneous exposure to either nitrogen mustard and carmustine (Fig. 5A) or nitrogen mustard and cisplatin (Fig. 5B). In contrast, when RR- or TLS-compromised cells were exposed to the same doses of drug combinations, a substantially greater-than-additive effect on cytotoxicity was observed (Fig. 5, A and B). These results demonstrate that, for a particular genetic background, simultaneous exposure to two drugs can be considerably more cytotoxic than the additive effects of single drug exposures.

#### Recombination Frequencies of DNA Damage-Processing Pathway-Compromised Strains in Response to Cisplatin and Nitrogen Mustard-Induced DNA Damage.

To examine further the role of RR in the processing of interstrand versus intrastrand DNA cross-links, we determined recombination frequencies for several DNA damage-processing-compromised strains. A second *lys2* allele (*lys2Δ3500*) was introduced into the relevant yeast strains (Jinks-Robertson and Petes, 1993), cells were exposed to cisplatin and nitrogen mustard, and recombination between the *lys2ΔBgl* allele located on chromosome II and the



**Fig. 4.** Cisplatin (A) and nitrogen mustard (B) sensitivities of MMR pathway-defective yeast strains are shown. Cells were exposed for 2 h to drug concentrations ranging from 5 to 400  $\mu$ M as described under *Materials and Methods*. Error bars represent the standard error for three experiments. Repair backgrounds of strains analyzed are indicated.



**Fig. 5.** Effects of drug combination treatments on survival of wild-type, RR-, and TLS-compromised strains. Wild-type or RR-compromised strains were exposed to single or combination treatments of nitrogen mustard and carmustine for 2 h as described under *Materials and Methods* (A). Wild-type, RR-, and TLS-compromised strains were exposed to single or combination treatments of nitrogen mustard and cisplatin for 2 h as described under *Materials and Methods* (B). Error bars represent the standard error for three experiments.

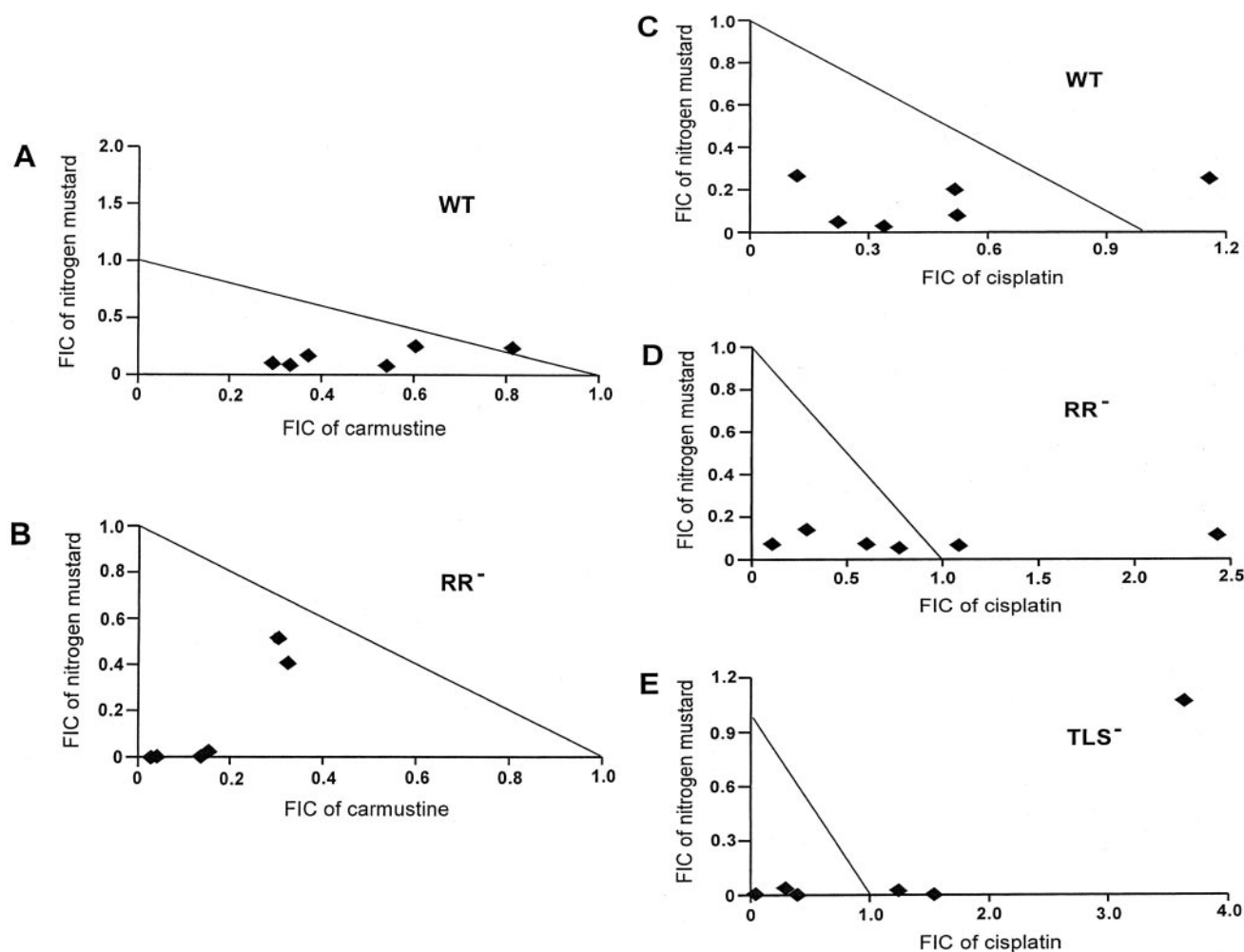
*lys2Δ3500* allele located on chromosome V was monitored by measuring the frequency of Lys<sup>+</sup> prototroph production. The induced recombination and mutation frequencies for the single and double pathway-compromised strains are presented in Fig. 7 and Table 2, respectively.

We established previously that several DNA damage processing-compromised *S. cerevisiae* strains show slight-to-moderate increases in spontaneous recombination frequencies compared with the wild-type strain (Swanson et al., 1999). When the same strains were exposed to cisplatin and nitrogen mustard, we observed significant increases in recombination frequencies, which were both drug- and strain-specific, compared with unexposed cells. Upon exposure to various concentrations of cisplatin, increases in recombination were observed for wild-type, NER-, TLS-, and NER/TLS-compromised strains, but the largest increases were observed for the wild-type and TLS-compromised strains (Fig. 7A). Exposure to higher concentrations of the drug (which decreased survival to about 1–5%) led to increases in recombination frequencies only for the wild-type and TLS-compromised strains, with no further increases in recombination frequency observed for the NER-compromised and NER/TLS-compromised strains (Fig. 7A). When the same strains were exposed to various concentrations of nitrogen mustard, an

increase in recombination was found for the wild-type, NER-, TLS-, and NER/TLS-compromised strains with the largest increase observed for the NER/TLS-compromised strain (Fig. 7B), suggesting that in the absence of NER and TLS, recombination is the main pathway for the processing of nitrogen mustard-induced lesions.

Substantial increases in recombination frequencies after cisplatin exposure in the wild-type and TLS-compromised strains, but only slight increases for the NER- and NER/TLS-compromised strains, indicate that Rad1p-mediated DNA nicking activity contributes to the recombination response to this drug. In contrast, substantial increases in nitrogen mustard-induced recombination frequencies observed for all four strains (including the NER-compromised strain) suggest that nitrogen mustard-induced DNA lesions do not require interaction with Rad1p before processing by recombination.

**Mutation Frequencies of DNA Damage-Processing Pathway-Compromised Strains in Response to Cisplatin or Nitrogen Mustard.** Increases in induced mutation frequencies upon exposure to cisplatin and nitrogen mustard were assessed by measuring the mutation frequencies at the *CAN1* locus (Tishkoff et al., 1997). Modest increases in mutation frequencies were observed for both cisplatin and nitrogen mustard in the wild-type strain (6- to 11-fold) and for



**Fig. 6.** Isobologram analysis of toxicity for various molar ratios of nitrogen mustard and carmustine combinations or nitrogen mustard and cisplatin combinations. Strains examined are indicated. The solid line indicates an isobole where drugs act additively and independently. Points above this line indicate antagonism between drugs, and points below this line indicate synergy. FIC, fractional inhibitory concentration.



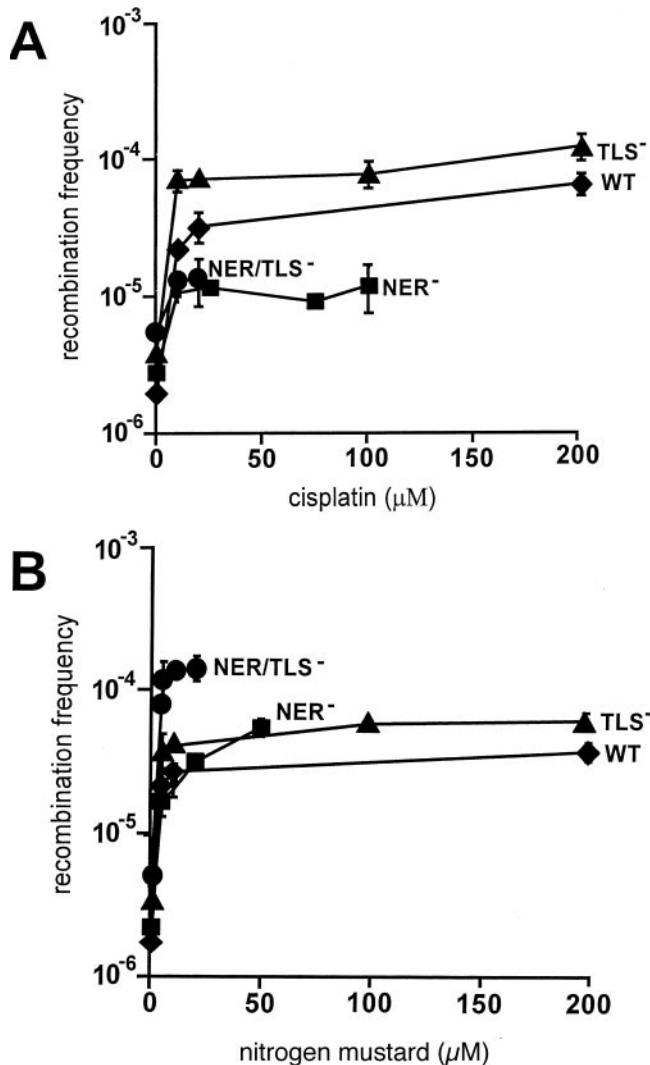
nitrogen mustard in the case of the NER-compromised strain (approximately 6-fold) (Table 2). The RR-compromised and NER/RR-compromised strains showed only slight increases in mutation frequencies (about 1.4-fold), even at survival levels of less than 1% (data not shown). As expected, no increases in mutation frequencies were found for the TLS-

compromised strain, which supports the idea that increases in mutation frequencies in our system are caused exclusively by DNA polymerase  $\zeta$ -mediated bypass of DNA lesions (Nelson et al., 1996).

**Cell Cycle Arrest Patterns in Response to Different DNA Cross-Linking Drugs.** Cell cycle analysis was performed by exposing asynchronous wild-type, NER-, RR-, and TLS-compromised strains to a drug concentration resulting in 5 to 10% survival. Arrest in the G<sub>2</sub>/M phase was observed when cells were exposed to cisplatin for the wild-type, RR-, and TLS-compromised strains (Fig. 8). However, cell cycle arrest was only temporary for the wild-type strain (3 and 4 h after release to growth media). When exposed to low concentrations of cisplatin (10 or 50  $\mu$ M), the NER-compromised strain was arrested in S phase. These results indicate that the DNA replication machinery can bypass cisplatin lesions in the absence of RR and TLS but not in the absence of NER. Similar to cisplatin, nitrogen mustard-induced arrest was observed at the G<sub>2</sub>/M phase for the wild-type, RR-, and TLS-compromised strains (Fig. 8). In contrast, arrest in the S phase was observed for the NER-compromised strain. Upon exposure to mitomycin, no cell cycle arrest was observed for the wild-type and TLS-compromised strains, whereas the NER-compromised strain was arrested in the G<sub>2</sub>/M phase and the RR-compromised strain was temporarily arrested in the G<sub>2</sub>/M phase at 2 and 3 h after release to growth media.

## Discussion

To gain insight into the cytotoxic effects of DNA cross-linking drugs, it is necessary to determine the events that affect their biological activities. Such events include cellular import and export, enzymatic activation and metabolism, binding to biological targets, and DNA damage processing. Yeast is an excellent model system for such studies because it is a simple, extensively studied eukaryotic organism for which many physiological processes are defined. A comprehensive study aimed at identification of *S. cerevisiae* genes that mediate sensitivity and resistance to several anticancer drugs has been published previously (Simon et al., 2000). However, that investigation used single-gene mutants only and reported drug doses resulting in 50% survival, thus limiting the interpretation of those experiments with respect to the capacity, specificity, or potential overlap of particular pathways. In contrast, we used 16 different single and multiple DNA damage-processing pathway-compromised strains of *S. cerevisiae* and exposed them to four different anticancer DNA cross-linking drugs under a wide range of drug concen-



**Fig. 7.** Cisplatin- (A) or nitrogen mustard-induced (B) recombination frequencies in single and double pathway-compromised strains. Cells were exposed for 2 h to various drug concentrations as described under *Materials and Methods* and plated onto media lacking lysine to select for recombinants. Error bars represent the standard error for at least three experiments. Repair backgrounds of strains analyzed are indicated.

TABLE 2

Induced mutation frequencies for cisplatin and nitrogen mustard treatments

Exposures that yielded between 1 and 5% survival were used to calculate mutation frequencies. The only exception was nitrogen mustard, where wild-type and TLS-compromised cells yielded 10 to 20% survival. Frequencies are expressed as recombinants or mutants per surviving cell. Numbers in parentheses indicate the average fold increases over control (untreated).

Relevant Genotype	Mutation Frequency ( $\times 10^{-6}$ )		
	Cisplatin	Nitrogen Mustard	Untreated Cells
WT	19.5 $\pm$ 3.7 (11)	13.3 $\pm$ 1.2 (6.5)	1.88 $\pm$ 0.18
NER-compromised	7.62 $\pm$ 3.8 (1.3)	36.9 $\pm$ 12 (6.4)	5.86 $\pm$ 0.32
REC-compromised	31.0 $\pm$ 0.90 (1.4)	29.2 $\pm$ 0.80 (1.4)	22.1 $\pm$ 0.97
TLS-compromised	0.955 $\pm$ 0.42 (0.87)	0.994 $\pm$ 0.36 (0.83)	1.20 $\pm$ 0.30
NER/REC-compromised	61.6 $\pm$ 17 (0.68)	133 $\pm$ 14 (1.5)	95.9 $\pm$ 16

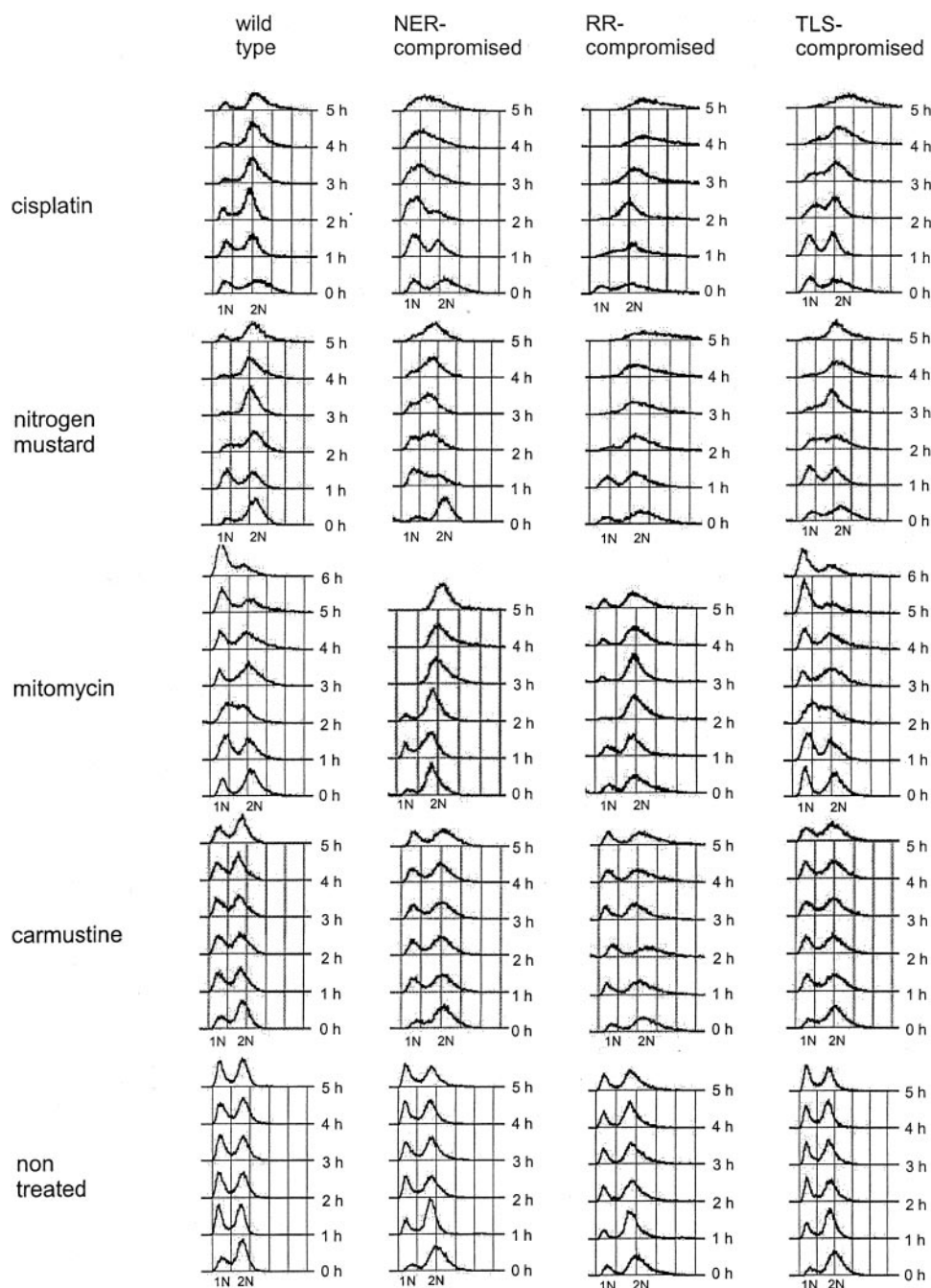
WT, wild type.



trations. This approach allowed us to determine the pathways crucial for toxic lesion processing upon exposure to each drug as well as the relative contribution of a pathway to cell survival.

In agreement with data published previously (Hannan et al., 1984; Simon et al., 2000; Grossmann et al., 2001), our results revealed that strains lacking NER, RR, and TLS individually were hypersensitive to cisplatin exposure (Fig. 3A), which indicates the involvement of all three DNA damage-processing pathways in the removal or bypass of toxic cisplatin-DNA lesions. Thus, each pathway is important sep-

arately in the survival of eukaryotic cells when exposed to cisplatin. However, when the NER pathway was functional but the RR and TLS pathways were compromised, cells exhibited extreme cisplatin sensitivity. These results were unexpected, considering the general belief that the NER pathway efficiently removes bulky lesions, such as those induced by cisplatin (Jamieson and Lippard, 1999). This finding supports the concept that RR and TLS may be mechanisms by which tumor cells can become resistant to this drug (Britten et al., 1999; Slupianek et al., 2001). Our results also suggest that RR and TLS process a majority of the toxic cisplatin



**Fig. 8.** Fluorescence-activated cell sorting analysis of asynchronous wild-type, NER-, RR-, and TLS-compromised strains exposed to cisplatin, nitrogen mustard, mitomycin, and carmustine. Cells were treated for 2 h, and cell cycle analysis was performed at 0, 1, 2, 3, 4, and 5 h after release to growth media as described under *Materials and Methods*. Cells in haploid or diploid states are indicated by 1N and 2N, respectively. An arrest in  $G_1$  is indicated by accumulation of cell population in 1N, arrest in  $G_2/M$  is indicated by accumulation of cell population in 2N, and arrest in S phase is indicated by the merging of the 1N and 2N peaks.

lesions and that having a functional NER system in conjunction with compromised RR and/or TLS systems does not result in adequate capacity for removal of toxic cisplatin lesions in the *S. cerevisiae* genome and eliminating toxicity.

In contrast to results published previously (Durant et al., 1999), we found that disruption of various MMR genes in *S. cerevisiae* had no effect on survival after exposure to cisplatin (Fig. 4A). One possible explanation for this difference is that we used shorter drug exposure times compared with Durant et al.

For nitrogen mustard (Fig. 3B), only the NER-compromised strain was moderately sensitized to the presence of DNA cross-links, and the TLS-compromised and RR-compromised strains were slightly more sensitive than the wild-type strain, exhibiting a plateau in cell survival at concentrations greater than 10  $\mu$ M. In contrast, all double pathway-compromised mutants were highly sensitized, with a greater-than-additive increase in sensitivity compared with their respective single mutants. Taken together, these data indicate cooperation between NER, RR, and TLS in the processing of nitrogen mustard-induced DNA cross-links and suggest the existence of at least two independent damage-processing pathways, one RR-dependent and one TLS-dependent, both of which require the participation of Rad1p when cells are exposed to high concentrations of nitrogen mustard.

Increases in recombination frequencies upon exposure to nitrogen mustard in the NER-compromised strain (Fig. 7) suggest the presence of some other factor(s) that can perform recombination-related DNA cleavage even in the absence of Rad1p. This observation is consistent with the recent finding that double-strand breaks (putative intermediates in inter-strand cross-link processing) were efficiently induced in NER-, BER-, and MMR-compromised strains upon exposure of *S. cerevisiae* to nitrogen mustard (McHugh et al., 2000).

Several different factors probably contributed to the observed differences in drug toxicities; these include intracellular concentration, metabolic processing, and kinetics of DNA binding as well as the types of structural deformations of the DNA helix induced by the four drugs used in this study (Fig. 1) (Dronkert and Kanaar, 2001). However, it is important to emphasize that, compared with the wild-type cells, double pathway-compromised strains were substantially more sensitive to the drugs used here. These findings suggest that the yeast strains used in this work should provide a useful screening system for molecules that can covalently bind to or cross-link DNA. The extent to which each of the five DNA damage-processing pathways considered in these studies (Fig. 2) participates in processing of DNA lesions

induced by cisplatin nitrogen mustard, mitomycin, and carmustine is presented in Table 3.

Measurement of both mutation (Table 2) and recombination frequencies (Fig. 7) also revealed differences in the processing of lesions induced by cisplatin and nitrogen mustard. The fold increases in recombination frequencies observed for cisplatin-exposed cells were the highest for wild-type and TLS-compromised strains, and a slight increase was found in the NER-compromised strain, indicating the importance of cisplatin-damaged DNA processing by NER before recombination. This finding can explain our observation that although the NER-compromised strain is sensitive to cisplatin, the NER pathway alone cannot protect cells against toxic cisplatin lesions. It is very likely that the elimination of Rad1p not only inactivates the NER pathway but also significantly compromises RR-mediated processing of toxic cisplatin lesions.

We also found that cell cycle arrest is both strain- and drug-specific (Fig. 8). For example, the NER-compromised mutant arrested in the S phase upon exposure to cisplatin or nitrogen mustard and in the G<sub>2</sub> phase upon exposure to mitomycin. These data indicate that cells cannot complete DNA replication in the presence of cisplatin- and nitrogen mustard-induced DNA adducts. In contrast, mitomycin-induced DNA adducts are apparently less of an obstacle, and cells are able to complete DNA replication followed by a temporary arrest at G<sub>2</sub>. In agreement with the observations from the cytotoxicity experiments, the RR-compromised strain arrested in G<sub>2</sub> after exposure to cisplatin, nitrogen mustard, and mitomycin, indicating that recombination is not absolutely required for cells to complete DNA synthesis. The differences in cell cycle arrest characteristics observed after exposure to the four DNA cross-linking drugs provides further support that cells process these DNA cross-link types differently, resulting in both strain- and drug-specific cell cycle arrest patterns.

These studies support the notion that observed differences in survival of single and multiple DNA damage-processing pathway-compromised strains can be used as a rapid, inexpensive, and efficient tool to study the mechanisms of action of anticancer drugs and reveal cellular pathways that process the types of DNA damage they cause. In addition, these strains may be useful for identifying potentially effective drug combinations.

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TABLE 3

Involvement of DNA damage-processing pathways (or pathway components) in response to DNA cross-linking anticancer agents

Cross-Linking Drug	DNA Damage-Processing Pathway				
	NER	RR	TLS	MMR	BER
Cisplatin	+++	+++	+++	0	0
Nitrogen mustard	+++	++	++	0	0
Mitomycin	++	++	++	N.D.	N.D.
Carmustine	+	+	++	N.D.	N.D.

N.D., not determined; 0, sensitivity equivalent to WT strain; +, sensitivity of the single DNA damage-processing pathway-disrupted strain is equivalent to the wild-type strain, but greater sensitivity is observed for strains simultaneously disrupted in this and some other pathway; ++, sensitivity of the single DNA damage-processing pathway-disrupted strain is moderate compared with the wild-type strain. Sensitivities of the multiple DNA damage-processing pathway-disrupted strains are moderate to high; +++, sensitivity of the single DNA damage-processing pathway-disrupted strain is high compared with wild-type strain. Sensitivities of the multiple DNA damage-processing pathway-disrupted strains are extreme.

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