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Utilization of *Saccharomyces cerevisiae* DNA damage management mutants for the determination of the mechanism of action of DNA targeting anticancer agents

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An abstract of a thesis submitted to the Faculty of the Graduate School of Emory University in partial fulfillment of the requirements for the degree of Master of Science in the
Graduate Division of Biological and Biomedical Science
Biochemistry, Cell and Developmental Biology
2009

Abstract

Utilization of *Saccharomyces cerevisiae* DNA damage management mutants for the determination of the mechanism of action of DNA targeting anticancer agents

By Bryn S. Moore

Reactive oxygen species (ROS) and DNA damage are implicated in the development and progression of cancer and in the resistance mechanism to chemotherapeutics. ROS are endogenously produced in the cell during oxidative phosphorylation and function in many signaling pathways. However, excessive ROS can lead to damage of DNA, proteins, and lipids. Cancer cells produce large amounts of ROS and accumulate oxidative DNA damage. Several DNA damage management pathways exist to remove or tolerate the presence of damaged DNA. The DNA repair pathways that process oxidative DNA damage include base excision repair (BER), nucleotide excision repair (NER), translesion synthesis (TLS), and recombination repair (REC). Here, a cytotoxicity assay was employed to screen anticancer drugs against a panel of isogenic DNA repair deficient yeast (*Saccharomyces cerevisiae*) to determine the pathways involved in processing damage induced by anticancer drugs. Additionally, a flow cytometry assay revealed that ROS was increased in all strains upon exposure to the anticancer agent cisplatin. In addition, DNA damage to nuclear DNA results in an increase in superoxide that may be due to the monoadducts that both cisplatin and transplatin form. However, the complex bifunctional DNA adducts that cisplatin forms could cause the cytotoxicity that makes cisplatin a successful anticancer agent. These assays have the potential to uncover the modes of action of DNA targeting antitumor agents and may provide information relevant to design of cancer chemotherapy treatment regimens as well as predicting responses in patients.

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Chapter 1

Introduction and Background

Reactive Oxygen Species. Reactive oxygen species (ROS) are oxygen-containing molecules that possess reactive properties and include singlet oxygen ($^1O_2$), superoxide ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) (Pelicano et al., 2004). In humans, the endogenous sources of ROS generation include mitochondrial oxidative phosphorylation (Brand, 1990; Nicholls, 18974), peroxisomes (Fahl et al., 1984), the cytochrome p450 system (Salvador et al., 2001) and cells involved in the inflammatory response (Babior, 1984; Wheeler, 2003). In addition to the endogenous sources of ROS, the generation of ROS within the cell can occur through exposure to exogenous agents such as UV and ionizing radiation (Costa and Moradas-Ferreira, 2001; Finkel and Holbrook, 2002). At normal levels, ROS are involved in cell signaling events including apoptosis, cell proliferation and cell cycle progression (Finkel, 1998; Fruehauf and Frank L. Meyskens, 2007; Kopnin et al., 2007; Kuo and Savaraj, 2006; Vafa et al., 2002). If unregulated, ROS pose a threat to cellular integrity through damaging lipids, protein, and nucleic acids as well as stimulating abnormal signaling (Cooke et al., 2003; Kopnin et al., 2007; Vafa et al., 2002). Each human cell is estimated to undergo 10,000 oxidative DNA damage events every day (Beckman and Ames, 1997). These base damages, if not repaired, can lead to mutations in DNA in replicating cells, and erroneous transcripts in all cells (Cooke et al., 2003; Karihtala and Soini, 2007; Kuo and Savaraj, 2006; Lambeth, 2004; Saxowsky et al., 2008). ROS can also stimulate cellular proliferation and genomic instability (Kopnin et al., 2007; Vafa et al., 2002). Taken together, the ability of ROS to

damage macromolecules and stimulate cellular proliferation and genomic instability makes unregulated ROS dangerous to the cell and to the organism. ROS are implicated in the etiologies of several human pathologies including cancer (Dreher and Junod, 1996), hypertension (Alexander, 1995), and aging-related disorders (Harman, 1981). Due to the deleterious effects of excess ROS, the cell has evolved defenses to protect against ROS-induced stress including ROS scavenging enzymes and DNA repair and damage tolerance pathways.

DNA damage management pathways. In *S. cerevisiae* there are four major DNA damage management pathways that are highly conserved and function to process oxidative DNA lesions (Friedberg *et al.,* 2006). These pathways include base excision repair (BER), nucleotide excision repair (NER), translesion synthesis (TLS), and recombination repair (REC) (Swanson et al., 1999). The BER machinery recognizes and repairs damaged bases, single strand breaks (SSBs) and AP (apurinic/ apyrimidinic) sites. In the first step of BER, a damaged base is removed by an N-glycosylase (for example, Ntg1 and Ntg2) and results in the production of an AP site (Figure 1.1A). Next, an endonuclease (e.g. Apn1) or an AP lyase (e.g. Ntg1 or Ntg2) cleaves the sugar phosphate backbone immediately adjacent (5'- or 3') to the AP site. If the sugar phosphate backbone was cleaved by the AP lyase, additional trimming is needed in order to process the SSB. Finally, DNA polymerase fills in the SSB by either short or long patch polymerization and the DNA is ligated, resulting in restoration of the original sequence (Friedberg *et al.,* 2006). NER can occur globally or coupled with transcription to process bulky helix-distorting DNA lesions (Friedberg et al., 2006). In NER a section of DNA

containing the damage is excised by cleaving the backbone 5' (by Rad1/Rad10 complex) and 3' (by Rad2) to the lesion (Figure 1.1B). DNA is resynthesized using the complementary strand as a template and ligated, which results in repaired DNA (Friedberg et al., 2006). The TLS pathway bypasses bulky lesions by utilizing low fidelity polymerases (Rev1 or Rev3) that take over replication and bypass the lesion, leading to mutations (Friedberg et al., 2006) (Figure 1.1C). REC is a DNA damage repair/tolerance pathway responsible for processing double strand breaks (DSBs). In the REC pathway, the DNA on either side of the DSB is resected by exonucleases, leaving 3' single stranded DNA (ssDNA) overhangs (Figure 1.1D). The 3' ssDNA invades a homologous sequence and DNA polymerases use the complementary sequence as a template to extend the 3' end. The non-invading 3' overhang is captured to form an intermediate with two Holliday junctions (HJs). DNA is synthesized and ligated, and the HJs are resolved with or without crossover (Krogh and Symington, 2004; Kuzminov, 2001).

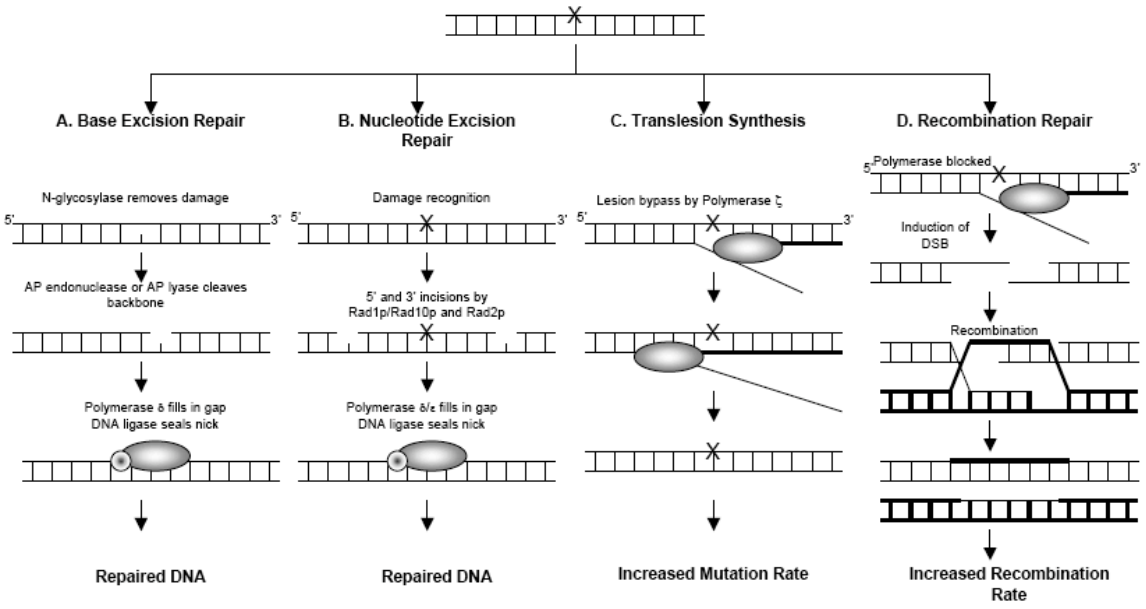
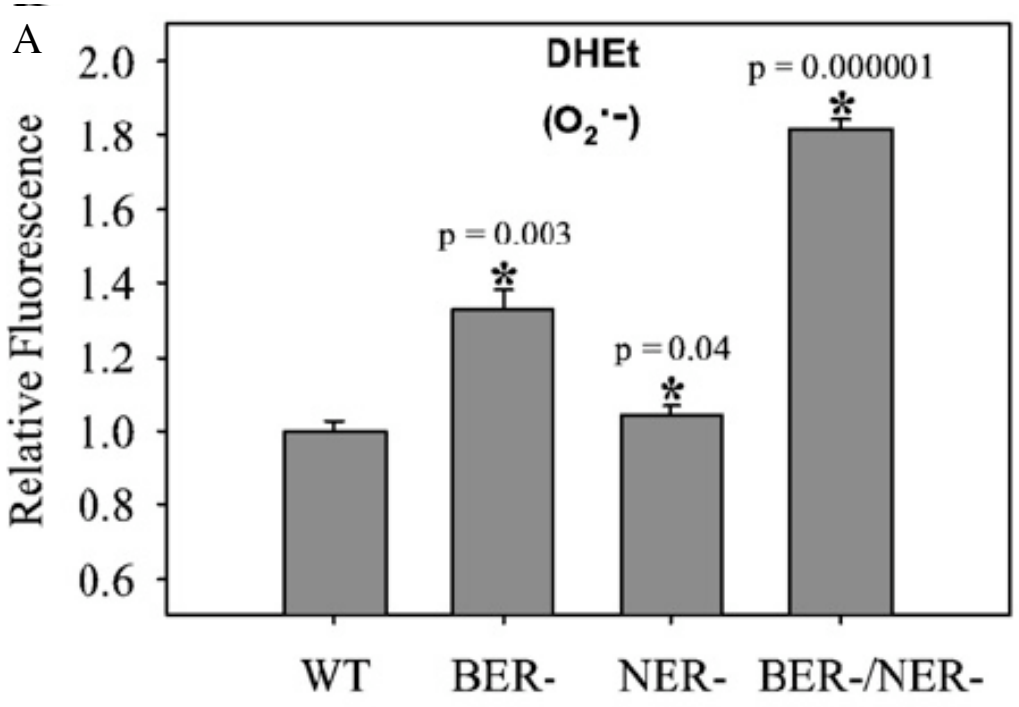


Figure 1.1. Model of the DNA damage management pathways responsible for processing oxidative DNA damage in *Saccharomyces cerevisiae*. A) Base excision repair removes small lesions resulting in repaired DNA. B) Nucleotide excision repair removes bulky lesions resulting in repaired DNA. C) Translesion synthesis bypasses lesions by utilizing low fidelity polymerases resulting in damage tolerance. D) Recombination repair utilizes homologous recombination to repair and/or tolerate damage (adapted from (Swanson et al., 1999).

DNA damage induces the production of ROS. Several lines of evidence suggest that DNA damage from endogenous and exogenous sources increases the intracellular concentration of ROS (Evert et al., 2004; Rowe et al., 2008; Salmon et al., 2004). The superoxide levels of DNA repair proficient and deficient strains determined using the fluorescent probe dihydroethidium and detected by flow cytometry showed that endogenous DNA damage resulting from inefficient repair consequently results in an increase in $O_2^{\bullet-}$ (Figure 1.2A). $O_2^{\bullet-}$ are produced in response to various types of DNA

damage including alkylating and UV-induced (Rowe et al., 2008; Salmon et al., 2004) (Figure 1.2B and 1.2C). The production of ROS in response to various types of DNA damage suggests that an increase in ROS is part of a general genotoxic response to DNA damage.



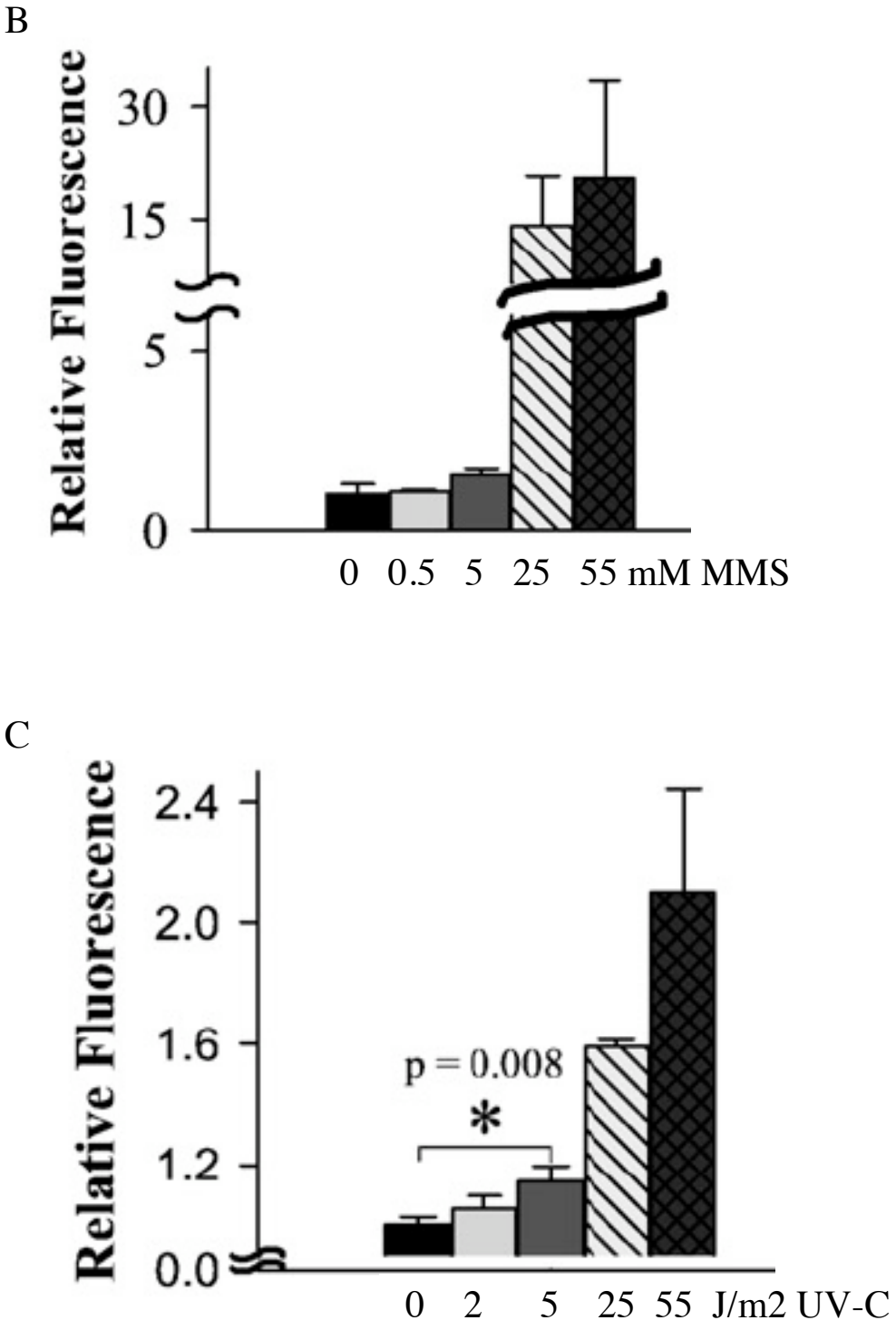


Figure 1.2. Superoxide production in response to various types of DNA damage (Rowe et al., 2008). A) Superoxide levels are increased in the cells of repair-compromised strains BER- (BER compromised), NER- (NER compromised), and BER-/NER- (BER-/NER compromised)

compared to wildtype (WT). B) WT cells have an increase in superoxide levels when exposed to the methylating agent methyl methane sulfonate. C) Superoxide levels are increased in WT cells when exposed to UV-C.

ROS and DNA damage involvement in cancer development, progression and treatment. Excess ROS can damage DNA (Cooke et al., 2003), as well as stimulate cellular proliferation and genetic instability (Kopnin et al., 2007; Vafa et al., 2002), which are all important steps in the development and progression of cancer (Hanahan and Weinberg, 2000; Lengauer et al., 1998; Luo et al., 2009) (Figure 1.3). Cancer cells produce large amounts of H_2O_2 and display an increase in hydroxyl radical-induced DNA damage (Malins et al., 1996; Szatrowski and Nathan, 1991). Additionally cancer cells can overcome the anti-proliferative effects of DNA damage (Luo et al., 2009). Not only do the pathways that generate or scavenge ROS and manage DNA damage play a role in the development and progression of cancer, they may also play a role in the efficacy of anticancer drug treatment. Because cancer cells have an increase in ROS levels (Malins et al., 1996; Szatrowski and Nathan, 1991) and DNA damaging agents can also induce an increase in ROS (Siomek et al., 2006), potential mechanisms of resistance to DNA damaging anticancer drugs include 1) increasing ROS-induced cell proliferation, 2) evading apoptosis, 3) modulating the drug target, 4) increasing the activity of the DNA damage management pathways that allow cells to survive treatment or 5) other mechanisms to potentially adapt to chronically elevated levels of ROS. For example, increases in the activity of NER and TLS in human cells can lead to resistance to the DNA cross-linking agent cisplatin (Ferry et al., 2000; Kelland, 2007; Lin et al., 2006; McGurk et al., 2006; Wu et al., 2004). However, the increase in ROS induced by

anticancer drugs may exacerbate the initial DNA damaging event by a feedback mechanism where the ROS oxidatively damage DNA.

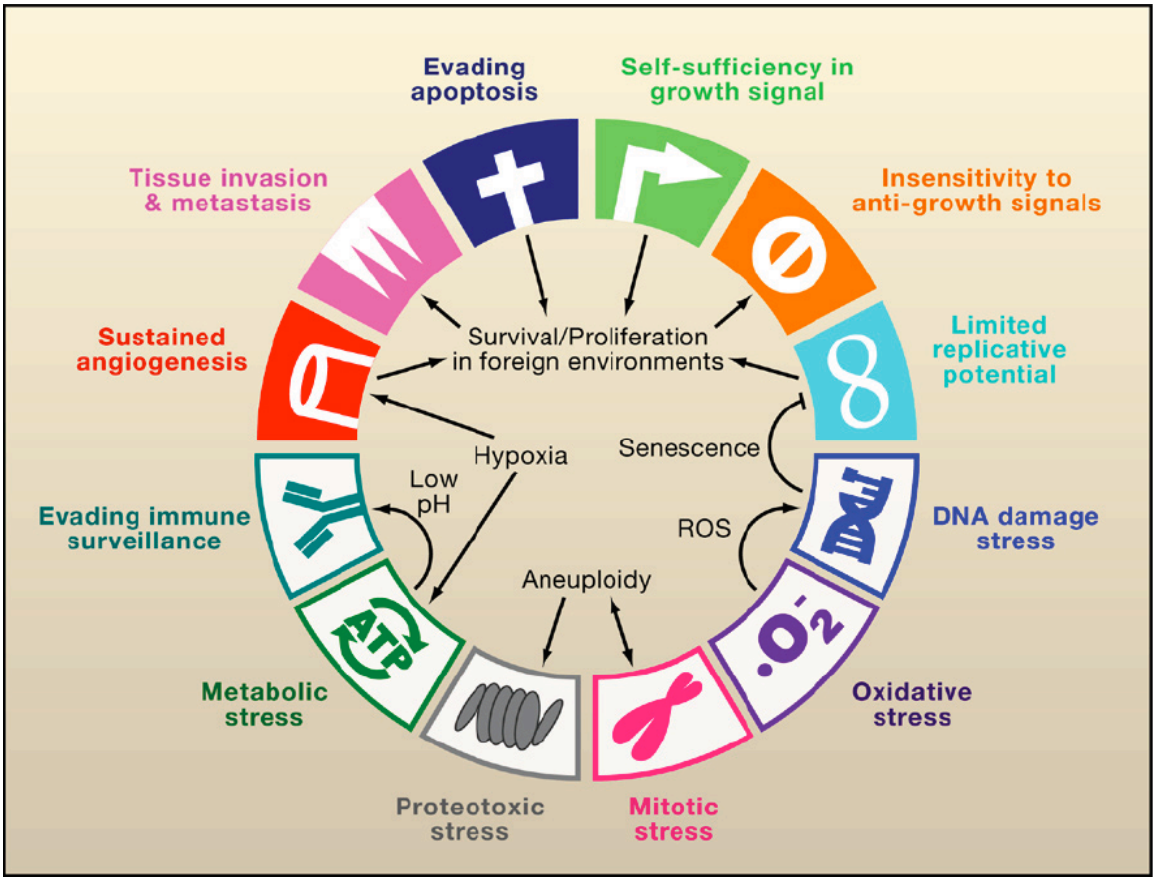


Figure 1.3. Hallmarks of cancer (revised). In addition to the six hallmarks proposed by Hanahan and Weinberg (top half), an additional six have been proposed including: evading immune surveillance, metabolic stress, proteotoxic stress, mitotic stress, oxidative stress, and DNA damage stress (Luo et al., 2009).

DNA Modifying Drugs

DNA damaging agents are commonly used in the treatment of cancer (Siddik, 2003). DNA damaging agents that prevent replication of DNA and induce apoptosis target

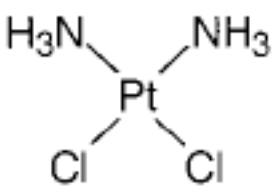
cancer cells specifically since cancer cells are rapidly dividing while most other adult cells are not. *Cis*-diamminedichloroplatinum (II), more commonly known as cisplatin, is used in the treatment of a number of cancers including ovarian, lung, bladder, testicular, and head and neck (Siddik, 2003). Cisplatin forms intra- and inter-strand cross-links with DNA, posing blocks to both transcription and replication (Boulikas and Vougiouka, 2004; Kelland, 2007) (structure seen in Figure 1.4A). *Trans*-diamminedichloroplatinum, or transplatin, an isomer of cisplatin (Figure 1.4B), also forms monoadducts with DNA; however, has been found to be clinically inactive. In addition to compounds that directly affect DNA, drugs that are able to modulate DNA by interacting with proteins are also popular anticancer agents. One such agent is Bix 01294, a drug in development for the treatment of cancer as a histone methyl transferase inhibitor. Bix 01294 specifically inhibits the histone methyl transferase G9a and prevents H3K9 dimethylation (structure seen in Figure 1.4C) (Kubicek et al., 2007). Though Bix 01294 does not directly damage DNA, the prevention of H3K9 dimethylation does have an effect on the chromatin state of the cell (Kubicek et al., 2007). Chromatin structure affects the ability of DNA damage management pathways to access and repair damage to DNA, therefore drugs that alter chromatin structure may play a role in sensitizing cells to anticancer agents (Morrison and Shen, 2009).

S. cerevisiae as an informative tool for gauging the effects of anticancer drugs

The budding yeast, *Saccharomyces cerevisiae*, has been employed as a simple eukaryotic model organism to study many processes including metabolic and signal transduction pathways (Simon and Bedalov, 2004). Many pathways are conserved from yeast to

humans, including the DNA maintenance mechanisms (Friedberg et al., 2006). The advantage of this yeast model system for gauging the effects of anticancer drugs is that key targets can be examined within the context of isogenic strains, which is not yet possible in human cells. Beljanski *et al*. used an isogenic panel of DNA damage management pathway deficient strains to screen DNA damaging agents. Using this unique panel, TLS, REC and NER were determined to be the pathways responsible for processing cisplatin-induced lesions (Figure 1.5). Prior to the Beljanski *et al*. study, only the NER pathway was attributed to processing cisplatin-induced DNA damage. Subsequently, evidence from Wu *et al*. implicated TLS in the processing of cisplatin-induced DNA lesions in human cells (Wu *et al*., 2004). The information gained from such studies in yeast can be directly translated and verified in studies with human cells.

A



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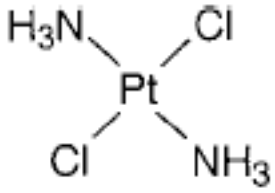

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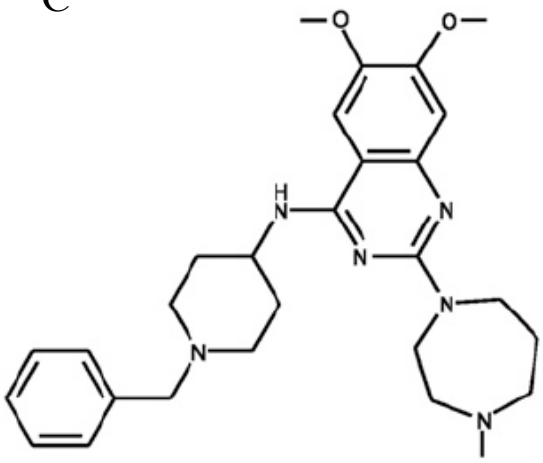


Figure 1.4. The structure of DNA modifying drugs (A) cisplatin, (B) transplatin and (C) Bix-01294.

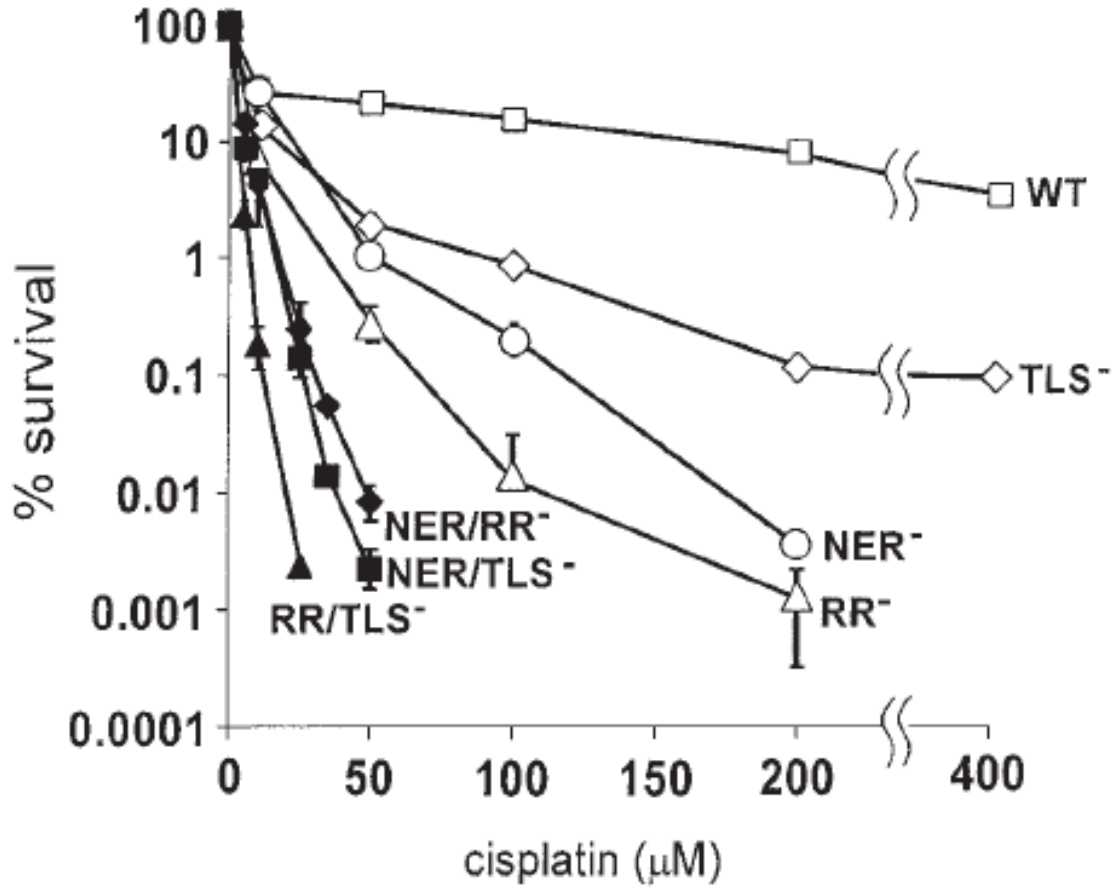


Figure 1.5. Sensitivity of DNA damage management-deficient *Saccharomyces cerevisiae* strains to cisplatin. *S. cerevisiae* cells were exposed to a range of doses of cisplatin and survival was determined as described in methods (Beljanski et al., 2004).

Conclusions

Reactive oxygen species (ROS) and DNA damage are implicated in the development and progression of cancer and in the resistance mechanism to chemotherapeutics. ROS are endogenously produced in the cell during oxidative phosphorylation and function in

many signaling pathways. However, excessive ROS can lead to damage of DNA, proteins, and lipids. Cancer cells produce large amounts of ROS and accumulate oxidative DNA damage. Several DNA damage management pathways exist to remove or tolerate the presence of damaged DNA. The DNA repair pathways that process oxidative DNA damage include base excision repair (BER), nucleotide excision repair (NER), translesion synthesis (TLS), and recombination repair (REC). Here, a cytotoxicity assay and a flow cytometry assay were employed to determine the mechanism of action of anticancer drugs in a panel of isogenic DNA repair deficient yeast (*Saccharomyces cerevisiae*).

Chapter 2

Utilization of *Saccharomyces cerevisiae* DNA damage management mutants for the identification of repair pathways relevant to established and developmental anticancer agent action.

Introduction

Here we used an isogenic panel of *Saccharomyces cerevisiae* strains, compromised in either one or two DNA damage management pathways (Table 2.1), to identify the pathways involved in processing damage induced by cisplatin or Bix 01294. Cisplatin is a frequently used anticancer agent that binds directly to DNA forming inter- and intra-strand cross-links. Bix 01294 is in development as an anticancer agent that inhibits the histone methyltransferase, G9a. Multiple isogenic pathway deficient strains allow us to determine overlap or competition among the various pathways. The ease and speed of using isogenic *S. cerevisiae* strains to identify key pathways involved in processing damage induced by each drug could prove to be a valuable tool to design combination therapies for immediate translation to mammalian cell studies and clinical trials.

Experimental Procedures

Cytotoxicity assay to screen anticancer drugs. The genotypes of the strains used in these studies are listed in Table 2.1. All strains with DNA repair defects in NER, BER, TLS, REC or any combination are isogenic derivatives of the wildtype (WT). Yeast strains were grown on YPD (1% yeast extract, 2% peptone, 2% agar, 2% dextrose, and 0.005% adenine sulfate) from frozen stocks (-80°C) for 2 days at 30°C. YPD liquid

was inoculated with one colony and grown for 12 hours at 30°C. The culture was used to inoculate 25 mL of YPD liquid. The yeast culture was grown to a density of approximately 2.0×10^7 cells/mL, pelleted and washed twice with dH_2O. Cells were resuspended in dH_2O, divided into 1 mL aliquots and exposed to different concentrations of xenobiotics for 2 hours at 30°C. Each drug was suspended in dH_2O to the desired concentration. Cells were washed with dH_2O and plated in duplicate dilutions to yield approximately 50-200 colonies per plate. After incubation for 2 days, colonies were counted. Finally, the percent survival for each drug exposure was calculated. The cytotoxicity assay was performed at least three times for each strain, drug and dose, and the percent survival for each condition was determined. The standard deviation for each strain at each dose was calculated and significance determined by a Student's t-test.

Table 2.1. Isogenic *S. cerevisiae* strains defective in DNA damage management pathways.

Strain	**Genotype**
Wildtype (WT)	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R*
BER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ1::HIS3*
NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG*
REC-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad52Δ::URA3*
TLS-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rev3Δ::kanR*
BER/NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ1::HIS3 rad1Δ::hisG*
NER/REC-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG rad52Δ::URA3*
TLS/NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG rev3Δ::kanR*
TLS/REC-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rev3Δ::kanR rad52Δ::URA3*
Rho^0	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rho^0*

Results

Sensitivities of DNA damage management deficient strains to cisplatin. REC, NER, and/or TLS compromised strains are sensitized to cisplatin, whereas the BER compromised strain is not (Figure 2.1). In order to determine the individual contribution of each DNA damage management pathway to the repair of cisplatin induced DNA lesions, strains deficient in two of these repair pathways were exposed to cisplatin and the cytotoxicity analyzed. The strains compromised in any combination of NER, REC, or TLS are highly sensitized to cisplatin (Figure 2.1). The BER-/NER- strain was slightly more sensitized to cisplatin than the NER- strain, but this is an additive effect of combining deficiencies in BER and NER.

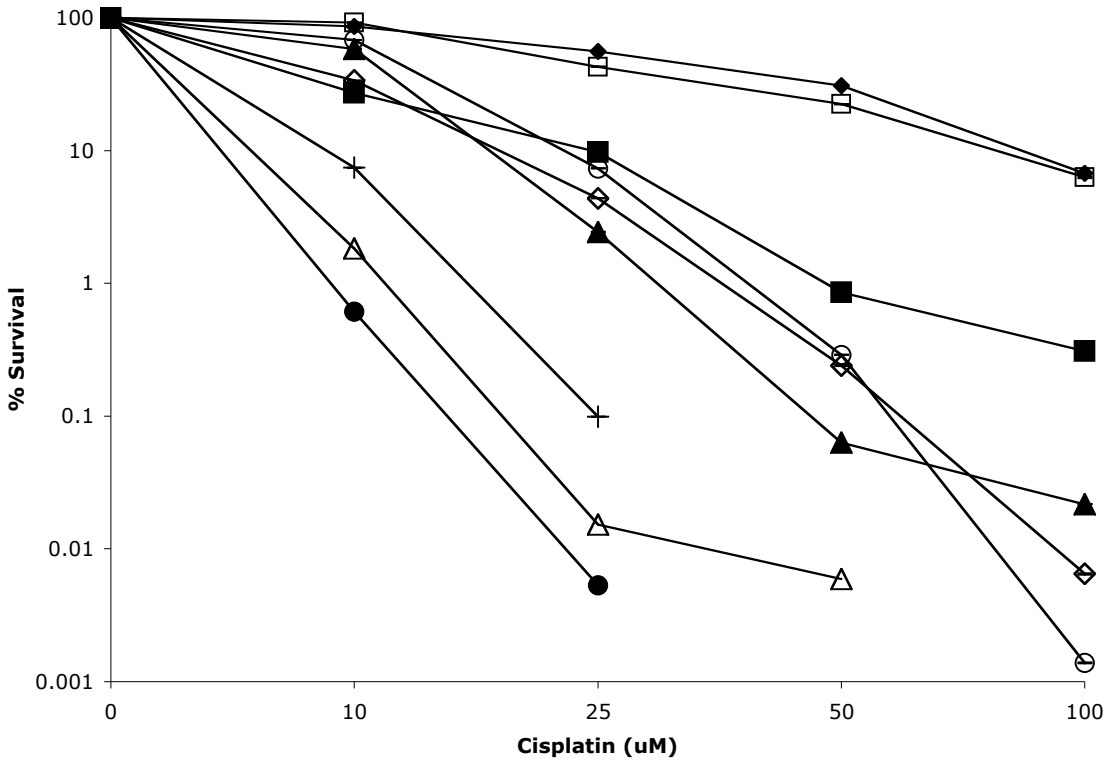
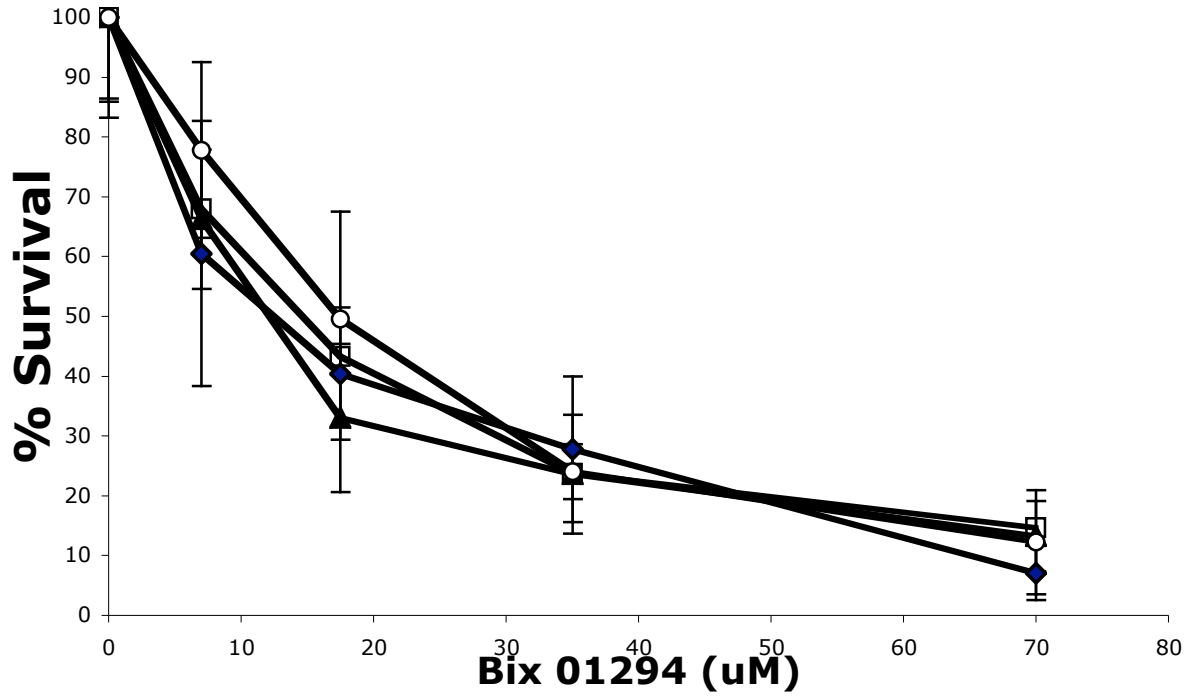


Figure 2.1. With the exception of the BER- (□) and wildtype (WT) (♦) *S. cerevisiae* strains, the DNA damage management-deficient strains NER- (▲), REC- (◇), TLS- (■), TLS-/NER- (△), NER-/REC- (+), TLS-/REC- (●) and BER-/NER- (○) are sensitized to cisplatin. Strains deficient in combinations of pathways are synergistically sensitive to cisplatin. Yeast cells were exposed to a range of doses of cisplatin and survival and standard deviation were determined as described in methods.

Sensitivities of DNA damage management deficient strains to Bix 01294. The DNA repair deficient strains show no increase in sensitivity to Bix 01294 at low doses as compared to wildtype cells (Figure 2.2). Likewise, the doubly deficient strains are not more sensitized than the WT or any singly deficient strain. However, at a higher dose of 200 μM Bix 01294, the BER-, NER-, and TLS- strains are modestly more sensitized than the WT strain (Figure 2.3). We conclude that the deficiencies in DNA damage management pathways will not have an effect on the efficacy of Bix 01294 at low doses and as a single agent.

A


% Survival
Bix 01294 (uM)
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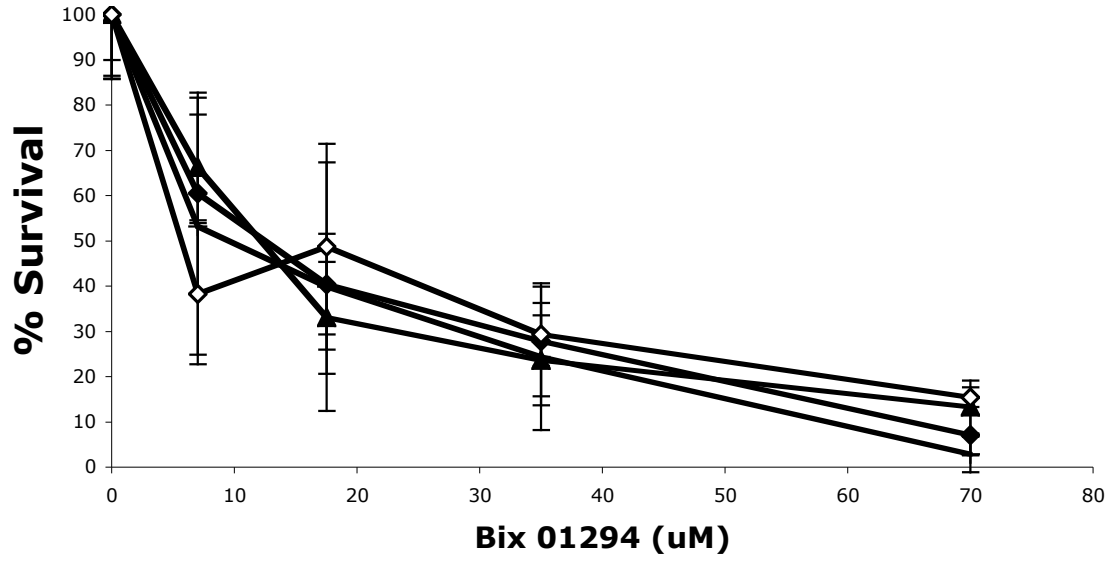
60
70
80
90
100

B


% Survival
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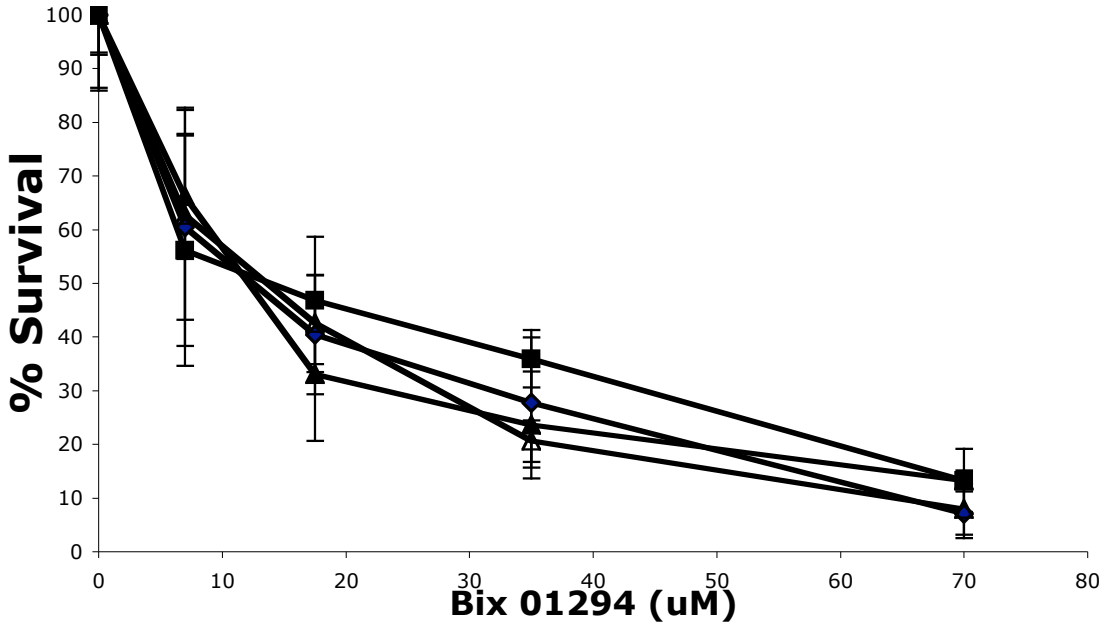
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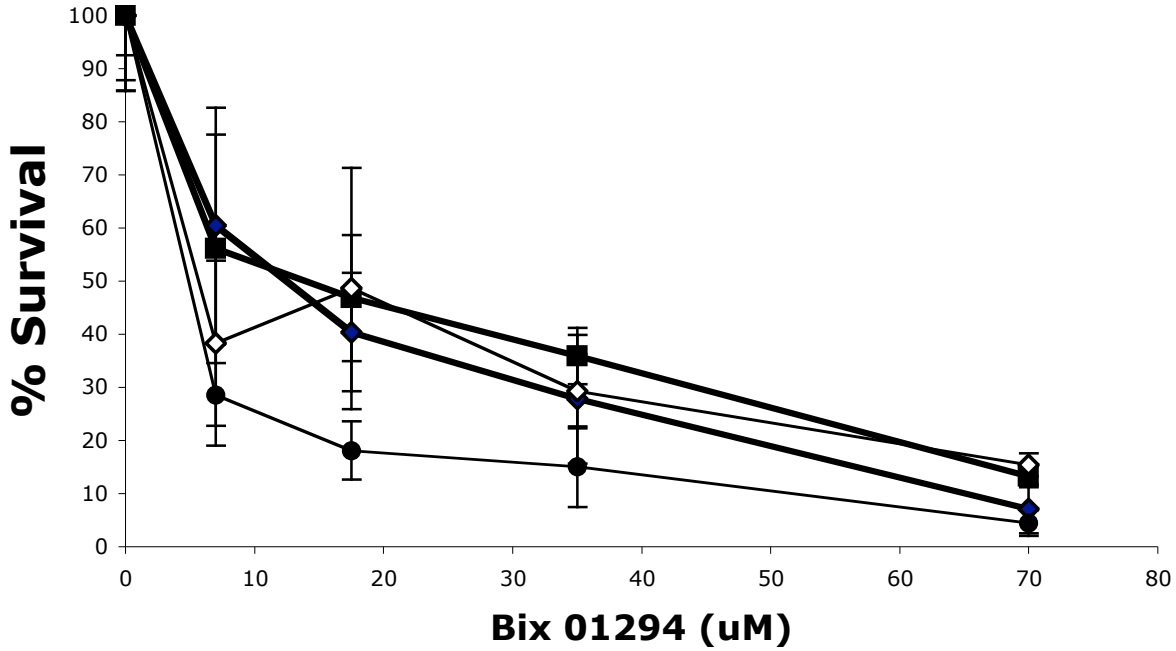
% Survival
Bix 01294 (uM)
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60
70
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90
100

Figure 2.2. DNA damage repair-proficient (WT) (♦) and -deficient (BER- (□), NER- (▲), REC- (◇), TLS- (■), TLS-/NER- (△), NER-/REC- (+), TLS-/REC- (●) and BER-/NER- (○)) *S. cerevisiae* strains are equally sensitized to Bix 01294. The *S. cerevisiae* strains A) WT, BER-, NER-, and BER-/NER-, B) WT, REC-, NER- and REC-/NER-, C) WT, TLS-, NER-, TLS-/NER-, D) WT, TLS-, REC-, REC-/TLS- were exposed to various doses of Bix 01294 and survival and standard deviation were determined as described in methods.

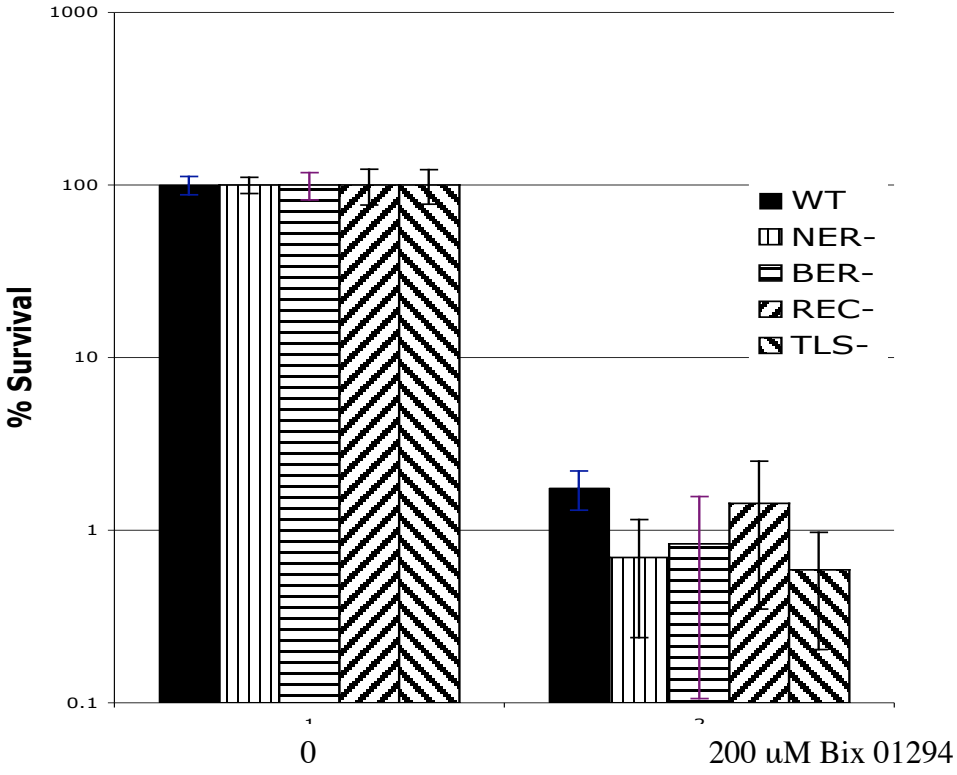


Figure 2.3. DNA damage management-deficient (BER-, NER-, TLS-) *S. cerevisiae* strains are sensitized to 200 μM Bix 01294, but the repair-proficient (WT) and REC-deficient strains are not. Yeast cells were exposed to various doses of Bix 01294 and survival and standard deviation were determined as described in methods.

Discussion

Sensitivities of DNA damage management deficient strains to cisplatin. NER, REC, and TLS defective strains are sensitized to cisplatin; suggesting that the NER, REC and TLS DNA damage management pathways are involved in the processing of cisplatin-DNA lesions. The BER defective strain was not significantly sensitized to cisplatin compared to WT. The BER-/NER- strain was additively sensitized to cisplatin, suggesting that the BER pathway is not involved in processing the cisplatin-DNA lesions, even when the NER strain is also compromised. TLS-/NER-, NER-/REC-, TLS-/REC- are exquisitely sensitized to cisplatin. In each of these strains, only one major DNA damage management pathway capable of processing cisplatin induced DNA lesions remains intact. In each case, the remaining DNA damage management pathway is not sufficient to confer significant survival, suggesting that NER, REC and TLS are all vital to the processing of cisplatin-DNA lesions and their combined activity on cisplatin-induced lesions is also important.

Sensitivities of DNA damage management deficient strains to Bix 01294. Bix 01294 is currently under development as an inhibitor of G9a, which dimethylates histone 3 at lysine 9 (Kubicek et al., 2007). None of the DNA damage management deficient strains were significantly more sensitized to Bix 01294 than the WT strain at low doses (10-70 μM). At high doses (200 μM) of Bix 01294 the NER, TLS, and BER strains are modestly more sensitized than WT. Though there was no sensitization of the DNA damage management system- deficient strains compared to WT, cytotoxicity was observed in all strains with even 10 μM Bix 01294. The yeast *S. cerevisiae* does not

possess the H3K9 histone mark, so the fact that this drug has any effect on yeast is somewhat surprising. This may indicate that Bix 01294 has another, undetermined effect on yeast cells, which may translate to mammalian cells. At higher doses of Bix 01294, there is a slight but significant sensitization of BER, NER and REC deficient strains. The cytotoxicity of Bix 01294 at high doses may be due to off target effects that may or may not include other histone methyltransferases.

Conclusions

TLS, REC and NER are involved in processing cisplatin-induced DNA lesions and one pathway alone is not sufficient to maintain survival. At lower doses of Bix 01294 (LD_0-LD_{90}) DNA repair proficient and deficient strains are equally sensitized to Bix 01294. At higher doses of Bix 01294 (LD_{99}) there is a modest increase in sensitivity of the BER-, TLS-, and NER- strains. Since yeast do not possess the histone mark H3K9me2, or a homolog to G9a, these results may suggest that Bix 01294 has an off target effect that may or may not be a histone methyltransferase.

Chapter 3

Chapter 3: Oxidative stress response to the *cis*-diamminedichloroplatinum (cisplatin)

Introduction

We employed an isogenic panel of *Saccharomyces cerevisiae* strains compromised in one or two DNA damage management pathways (Table 2.1) in order to examine the endogenous level of superoxide ($O_2^{\bullet-}$). Additionally, we assayed the superoxide level of these strains when exposed to the anticancer agent cisplatin. In an attempt to determine how and why the $O_2^{\bullet-}$ level was elevated in response to cisplatin exposure, we established that superoxide increased in the mitochondria, independent of the DNA damage. This is a novel finding with important implications in the mechanism of action of cisplatin.

Experimental Procedures

Cytotoxicity assay to screen anticancer drugs. Described in Chapter 2: Experimental Procedures.

Determination of ROS levels. The genotypes of the strains used in these studies are listed in Table 2.1. All strains with DNA repair defects in NER, BER, TLS, REC or any combination are isogenic derivatives of a wildtype (WT), DNA repair-proficient strain. Yeast strains were grown on YPD (1% yeast extract, 2% peptone, 2% agar, 2% dextrose, and 0.005% adenine sulfate) from frozen stocks (-80°C) for 2 days at 30°C. YPD liquid was inoculated with one colony and grown for 12 hours at 30°C. The culture was used to

inoculate 25 mL of YPD liquid. The yeast culture was grown to a density of approximately 2.0×10^7 cells/mL, pelleted and washed twice with dH_2O. Cells were resuspended in dH_2O, divided into 3 mL aliquots and exposed to different concentrations of xenobiotics for 2 hours at 30°C. Cells were pelleted and washed with dH_2O and resuspended in YPD liquid. A 1mL aliquot was removed for the cytotoxicity assay as described in Chapter 2 Experimental Procedures. The resuspended cells were incubated with the $O_2^{\bullet -}$-specific fluorescent probe dihydroethidium (DHEt) or Mitosox in the dark for 2 hours at 30°C. DHEt detects total cellular $O_2^{\bullet -}$ and Mitosox is specific for mitochondrial $O_2^{\bullet -}$. The cells were pelleted, washed with dH_2O and resuspended in PBS. 10,000 cells were analyzed for fluorescence using the BD™ LSR II flow cytometer (BD Biosciences) in each experiment. Each experiment was performed at least three times and the mean fluorescence for each experiment was determined and averaged. The standard deviation was calculated for each strain and drug dose and significance determined using a Student's t-test. The ROS response to drugs was determined by calculating the average fold increase of fluorescence compared to untreated cells for each strain. The endogenous levels of ROS for each strain were compared to the WT strain.

Results

Endogenous superoxide levels of DNA damage management deficient strains. To assess the effect of endogenous DNA damage on ROS, the level of superoxide was determined in the DNA damage management deficient strains. The strains defective in NER, TLS, BER, and TLS/NER displayed the same superoxide levels as compared to the WT strain. However, the superoxide levels of the REC-, TLS-/REC-, and NER-/REC- strains are

significantly increased above WT (Figure 3.1). The superoxide level of the BER-/NER- strain is also moderately increased above WT.

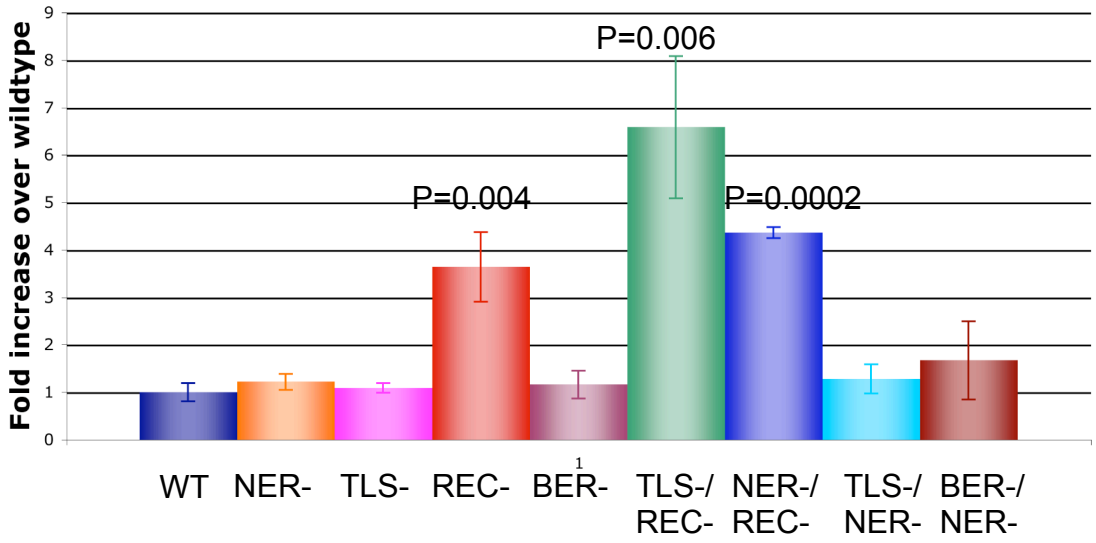
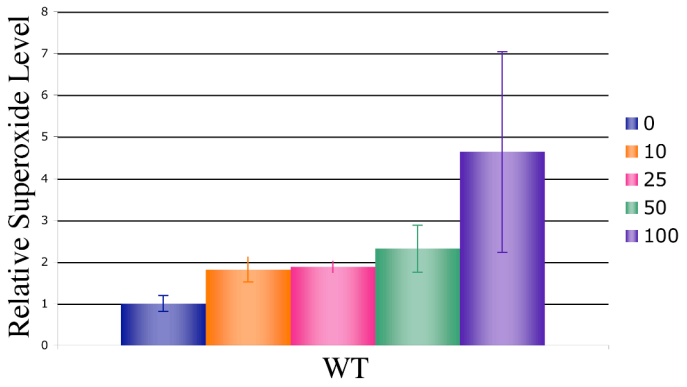


Figure 3.1. Untreated *S. cerevisiae* strains defective in the DNA damage management pathways REC, TLS/REC, and REC/NER display an increase in superoxide ($O_2^{\bullet -}$) compared to wildtype (WT). The $O_2^{\bullet -}$ levels were determined in WT and DNA damage management-defective strains by incubating with the fluorescent probe dihydroethidium and analyzing by flow cytometry.

Cisplatin induced superoxide levels in DNA damage deficient strains. To determine the effect an exogenous DNA damaging agent has on superoxide levels, the panel of DNA damage management defective strains was exposed to cisplatin and the superoxide levels were detected. Each strain exhibited a dose-dependent increase in the level of superoxide. Surprisingly, the strains that are sensitized to cisplatin cytotoxicity (NER-, REC-, TLS-, TLS-/NER-, NER-/REC-and TLS-/REC-) and the strains that are not sensitized to cisplatin (BER- and WT) display a marked increase over the relative baseline level (unexposed cells) of superoxide (Figure 3.2).


Relative Superoxide Level
0
1
2
3
4
5
6
7
8
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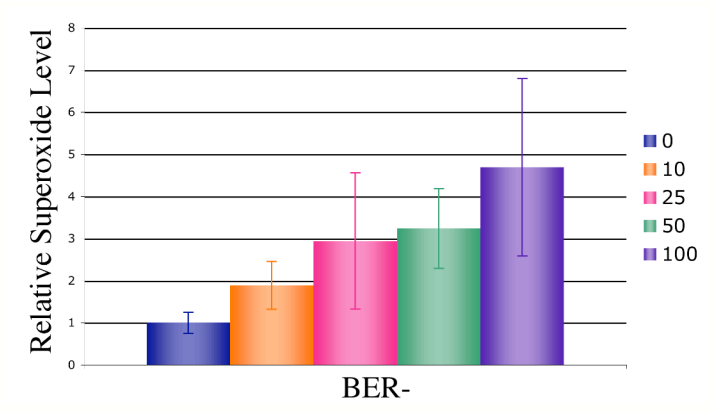
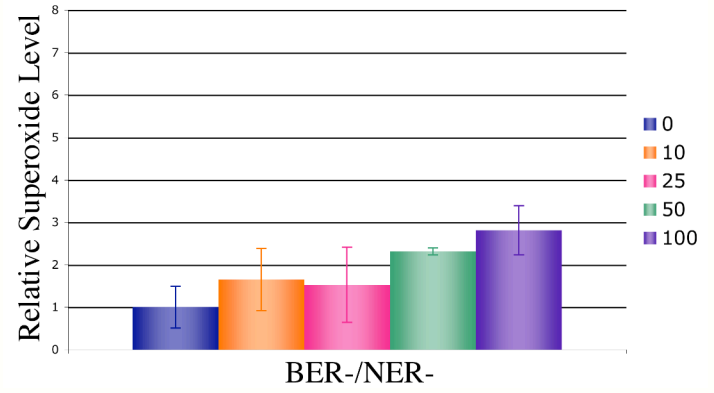
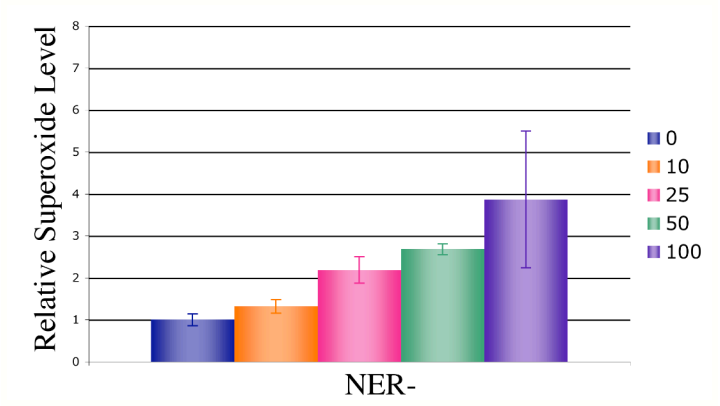
10
25
50
100
WT


Relative Superoxide Level
0
1
2
3
4
5
6
7
8
0
10
25
50
100
BER-


Relative Superoxide Level
0
1
2
3
4
5
6
7
8
0
10
25
50
100
BER-/NER-


Relative Superoxide Level
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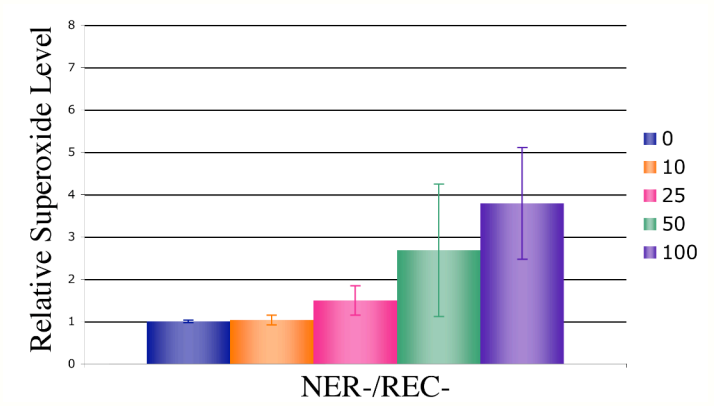
50
100
NER-


Relative Superoxide Level
0
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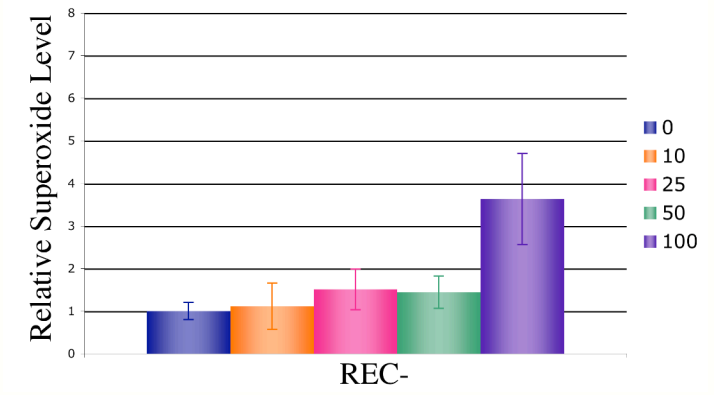
7
8
0
10
25
50
100
NER-/REC-


Relative Superoxide Level
0
1
2
3
4
5
6
7
8
0
10
25
50
100
REC-

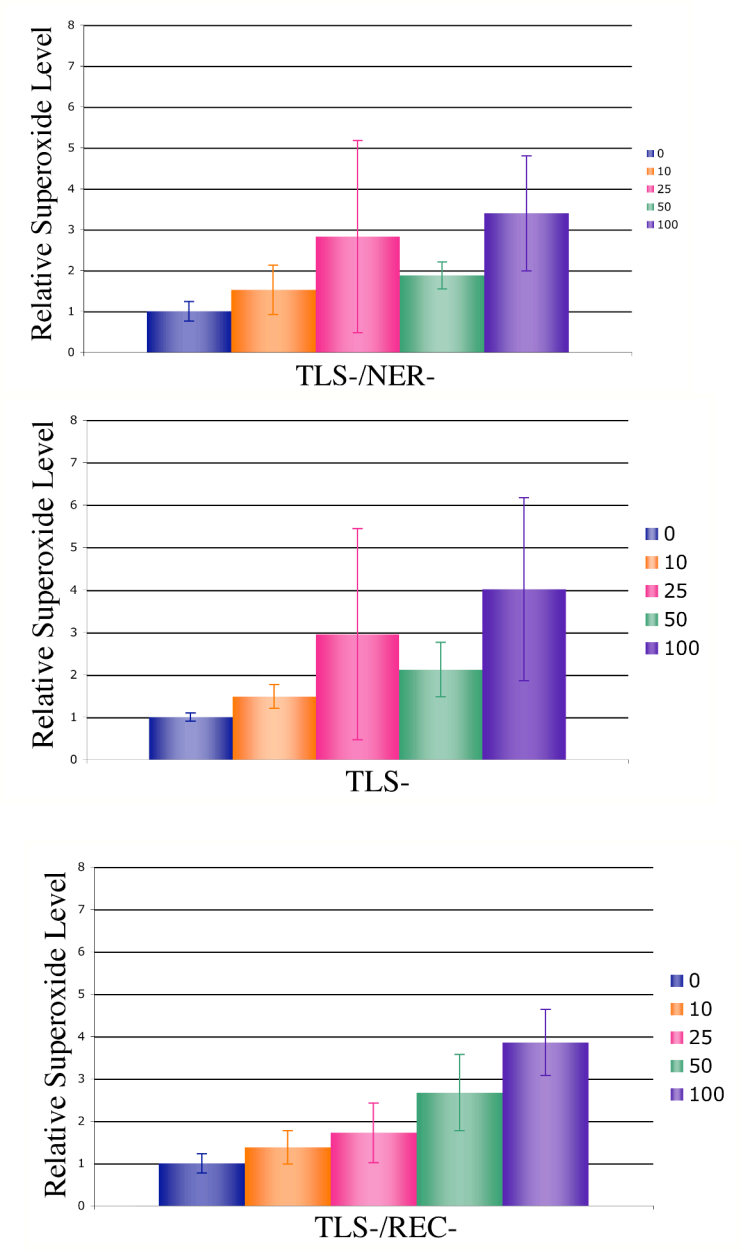


Figure 3.2. Cisplatin induces an increase in total cellular superoxide ($O_2^{\bullet -}$) in wildtype (WT) and DNA damage management-deficient *S. cerevisiae* strains. $O_2^{\bullet -}$ levels were determined by flow cytometry of cells incubated with dihydroethidium (DHEt). The $O_2^{\bullet -}$ levels of treated cells were normalized to untreated cells from each strain to yield the relative increase in $O_2^{\bullet -}$ with cisplatin treatment of 10, 25, 50, and 100 μM.

Origin of sub-cellular superoxide increase. In an attempt to determine the sub-cellular origin of the increase in superoxide in the yeast cells, we utilized two fluorescent probes dihydroethidium (DHEt) and Mitosox. Dihydroethidium detects the total superoxide in the cell, whereas Mitosox detects only mitochondrial superoxide. There is a dose-dependent increase in superoxide in both the WT and NER-deficient strain with each fluorescent probe (Figure 3.3).

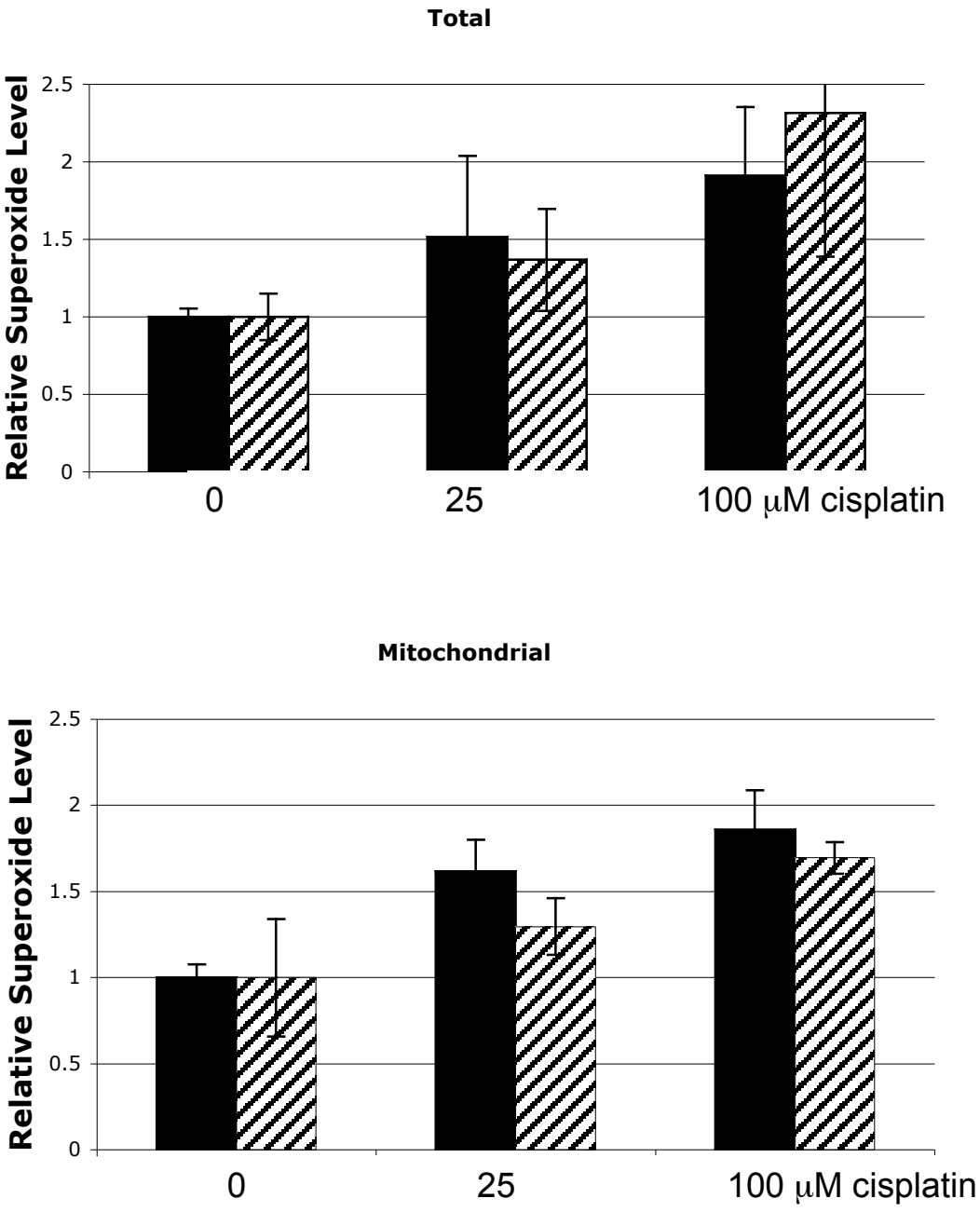


Figure 3.3. Cisplatin induces an increase in total cellular superoxide ($O_2^{\bullet -}$) (left) and mitochondrial superoxide (right) in wildtype (**WT**) (solid bars) and **NER** -deficient (striped bars) *S. cerevisiae* strains. The $O_2^{\bullet -}$ levels were determined by incubating with the fluorescent probe dihydroethidium or Mitosox and analyzing by flow cytometry. The $O_2^{\bullet -}$ levels of the treated samples were normalized to the untreated samples for each strain to yield the relative increase in $O_2^{\bullet -}$ with cisplatin treatment.

Mitochondrial DNA damage has a modest effect on the superoxide levels. Since mitochondria are a potential source of the increased superoxide, we desired to determine if the increase in superoxide was a result of an increase in mitochondrial DNA damage. For these experiments, we utilized two strains, the wildtype and a rho^0 strain that is devoid of mitochondrial DNA and is respiration defective. The rho^0 is modestly less sensitized to cisplatin than the control (Figure 3.4). However, each strain displayed the same increase in superoxide level in response to cisplatin (Figure 3.5).

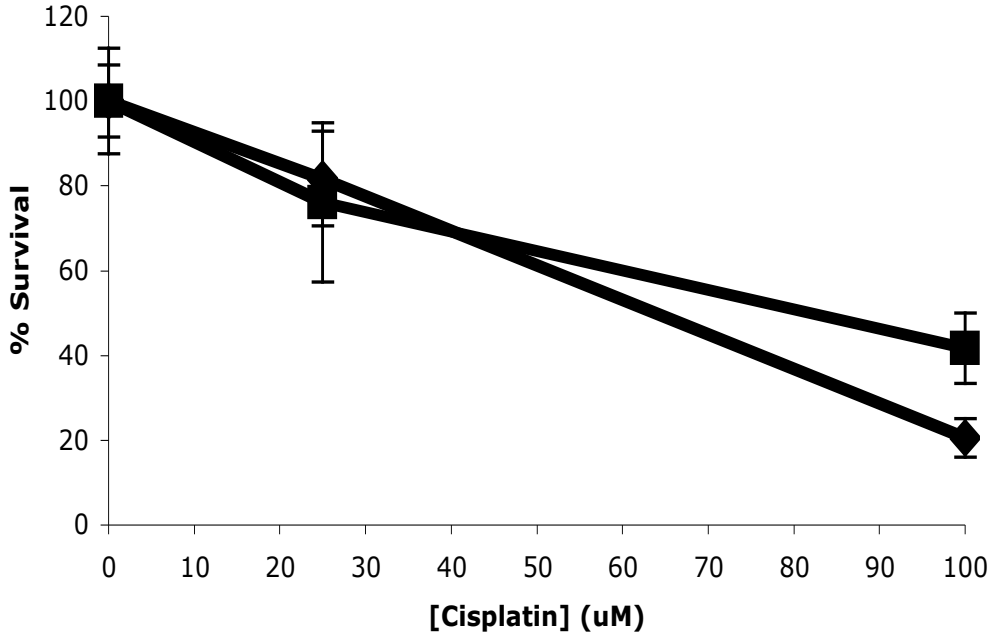


Figure 3.4. The mitochondrial DNA deficient strain (rho^0) (■) is less sensitized to high doses of cisplatin compared to rho$^+$ wildtype strain (WT) (♦). Yeast cells were exposed to a range of cisplatin doses and survival and standard deviation were determined as described in methods.

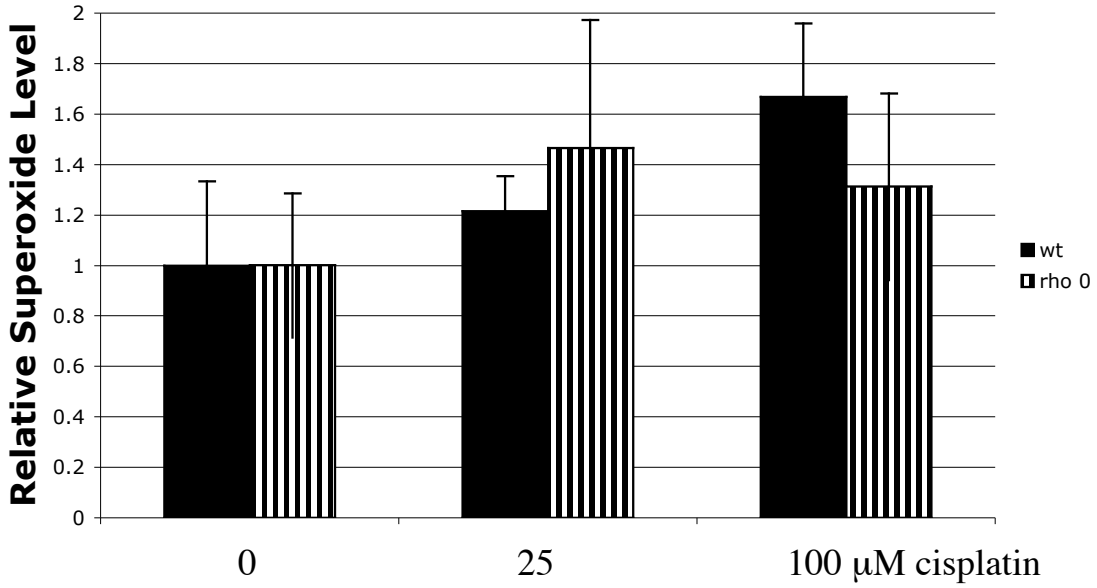


Figure 3.5. Cisplatin induces an increase in superoxide ($O_2^{\bullet -}$) in the rho$^+$ (**WT**) (solid bars) and non-functional mitochondria strain (**rho**^{0}) (striped bars). The $O_2^{\bullet -}$ levels were determined by incubating with the fluorescent probe dihydroethidium and analyzing by flow cytometry. The $O_2^{\bullet -}$ levels of the treated samples were normalized to the untreated samples for each strain to yield the relative increase in $O_2^{\bullet -}$ with cisplatin treatment.

<u>Transplatin increases superoxide levels similarly to cisplatin, despite being less cytotoxic</u>. Transplatin is an isomer of cisplatin that is capable of forming monoadducts with DNA. Transplatin, though clinically ineffective, induces modest cell killing in our studies. As previously reported, NER defective strains are slightly more sensitized to transplatin than wildtype strains (Wilborn and Brendel, 1989). Transplatin induces an increase in

superoxide in both the DNA proficient and deficient strains in a dose dependent manner (Figure 3.7) comparable with the response observed with cisplatin (Figures 3.2 and 3.3).

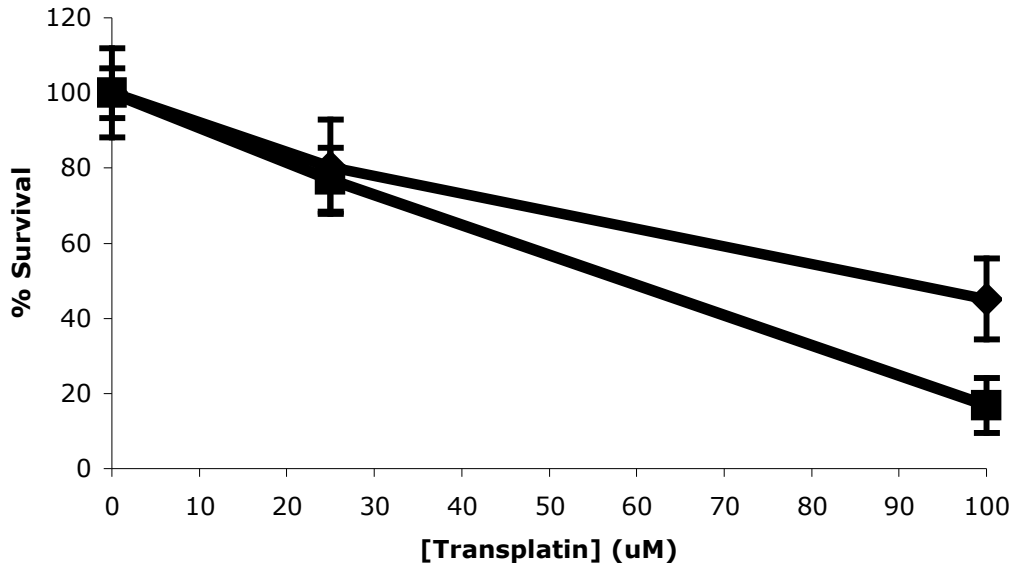


Figure 3.6. The DNA damage management-deficient *S. cerevisiae* strain NER- (■) is sensitized to transplatin treatment compared to wildtype (WT) (◆). Yeast cells exposed to a variety of doses of transplatin and survival and standard deviation were determined as described in methods.

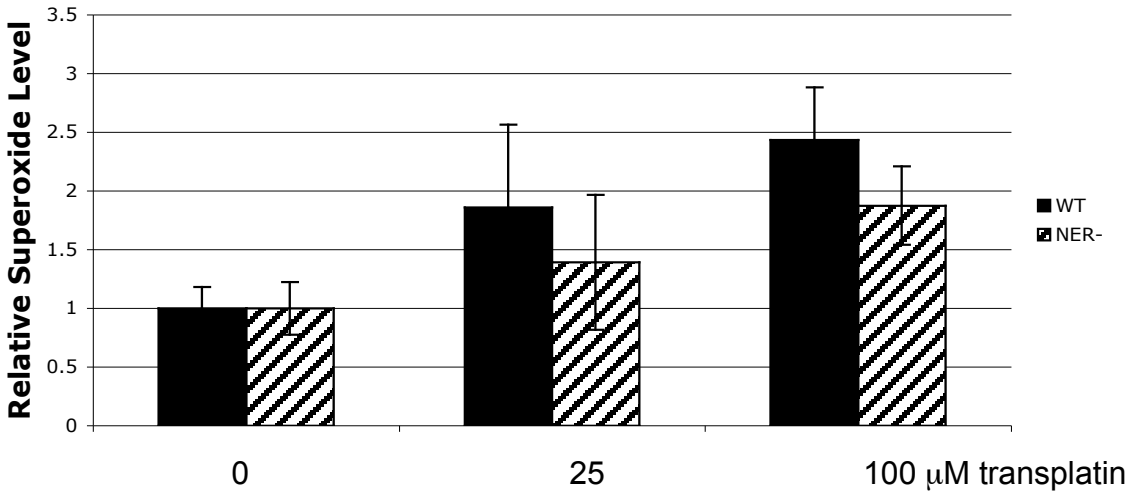


Figure 3.7. Transplatin induces an increase in superoxide ($O_2^{\bullet -}$) in both the wildtype (**WT**) and DNA damage management deficient strain (**NER-**). The $O_2^{\bullet -}$ levels were determined by

incubating with the fluorescent probe dihydroethidium and analyzing by flow cytometry. The $O_2^{\bullet -}$ levels of the treated samples were normalized to the untreated samples for each strain to yield the relative increase in $O_2^{\bullet -}$ with cisplatin treatment.

Discussion

Endogenous superoxide levels of DNA damage deficient strains. DNA damage management defects do not necessarily result in an increase in endogenous superoxide levels. This is reflected by the NER-, TLS-, BER- and TLS-/NER- strains displaying the same superoxide level compared to WT. In contrast, BER-/NER- (modest effect), REC-, NER-/REC-, TLS-/REC- strains have increased superoxide levels compared to repair-competent cells. Surprisingly, all strains that are REC deficient display an increase in superoxide. REC- cultures often contain grossly large cells among normal size cells (data not shown). In addition, REC-, NER-/REC-, and TLS-/REC- cultures grow more slowly than WT or other DNA damage management defective strains (data not shown). In a direct comparison of WT and REC- strains grown on YPD plates following growth in YPD liquid, it appears that the REC- strain does not grow as well on YPD, suggesting that more REC- cells are dying (data not shown). Though some DNA damage management defective strains display an increase in superoxide, some do not. This finding suggests that the DNA damage that results from deficiencies in one or two pathways may be handled by other pathways, or only certain types of DNA lesions are able to elicit an increase in superoxide. Though this hypothesis remains to be tested, the notion that only some types of damage may elicit this type of response is likely due to the data that suggests that transplatin induces an increase in superoxide level without the substantial cytotoxicity observed with cisplatin treatment (discussed below).

Cisplatin induced superoxide levels in DNA damage deficient strains. The strains that are sensitized to cisplatin cytotoxicity include NER-, TLS-, REC-, TLS-/REC-, NER-/REC-, and TLS-/NER-; however, the BER- strain is not sensitized. Despite the difference in the sensitivities to cisplatin, every strain examined had a cisplatin-dose dependent increase in superoxide level above the untreated level. The increase in superoxide presumably would result in oxidative DNA damage, which would be repaired by BER. However, the BER strain was not sensitized to cisplatin. These data may suggest that an increase in damage due to ROS levels may not be sufficient to observe differences between the cytotoxicity induced in WT and BER- strains. The cytotoxicity that cisplatin induces in mammalian cells has been attributed to the DNA damage the drug induces. Cisplatin appears to induce an increase in superoxide levels regardless of its cytotoxic effects.

Origin of the superoxide increase. The increase in superoxide levels in all strains when exposed to cisplatin is not dependent on the cytotoxic effects due to DNA damage. The DNA damage effects are assumed to be mostly nuclear, however we determined whether the superoxide originated in the mitochondria through using two different fluorescent dyes that detect total cellular (dihydroethidium) and mitochondrial (MitoSox) superoxide. Both dyes detected a cisplatin dose-dependent increase in superoxide in the WT and NER-defective strains. Though we cannot directly compare the levels between each dye, it is clear that at least one component of the superoxide increase is mitochondrial in origin.

Mitochondrial DNA damage has little effect on the superoxide levels. Since there was an increase in superoxide levels in mitochondria when cells were exposed to cisplatin, we determined the effect of mitochondrial DNA on cell viability and superoxide levels following exposure to cisplatin. Yeast cells can survive in the absence of mitochondrial DNA. Utilizing WT and rho^0 (mitochondrial DNA absent) cells, we determined the effect cisplatin-induced DNA damage has on mitochondrial DNA, cytotoxicity, and superoxide levels. Rho^0 cells are modestly less sensitized to cisplatin than the WT cells, suggesting that mitochondrial DNA damage has a modest effect on the total cytotoxicity elicited by cisplatin. In addition, the superoxide levels of both rho^0 and WT strains are comparable, suggesting that mitochondrial DNA damage makes little contribution to the superoxide effect of cisplatin. If DNA damage is the signal to increase superoxide levels, then this signal must be initiated from DNA in the nucleus, not the mitochondria.

Transplatin increases superoxide levels similarly to cisplatin, despite being less cytotoxic. Transplatin, a stereoisomer of cisplatin and clinically ineffective agent, forms monofunctional adducts with DNA, but not the stable, complex bifunctional structures that cisplatin can induce (Boudvillain *et al*., 1995). However, we decided to determine the effects transplatin has on both cytotoxicity and superoxide level. As previously reported, transplatin has a modest killing effect, which is increased in the NER- strain (Wilborn and Brendel, 1989). When exposed to transplatin, the superoxide levels of both the WT and NER- strain are also increased in a dose-dependent manner. As proposed in the above section "Endogenous superoxide levels of DNA damage deficient strains," this result may suggest that only certain types of DNA damage are able to elicit an increase in

superoxide levels. Both transplatin and cisplatin can form monofunctional adducts. However, only cisplatin can form the stable bifunctional intra- and inter-strand adducts (Boudvillain *et al*., 1995, Millard and Wilkes, 2000). Since the superoxide level is increased with exposure to both trans- and cisplatin, perhaps the increase is due to the monofunctional adducts formed in the nuclear DNA; whereas, the cytotoxicity that is observed in cells exposed to cisplatin is due to the bifunctional intra-strand adducts that only cisplatin is capable of forming. Perhaps the signal from the damaged nuclear DNA is sent throughout the cell and multiple systems respond to increase the level of superoxide.

Conclusions

Cisplatin induces an increase in superoxide levels that is independent of DNA repair capacity. Cisplatin induces DNA damage that directly affects cytotoxicity. Transplatin induces little cytotoxicity while inducing large increases in superoxide levels. These results suggest that cisplatin induces DNA damage and an independent increase in superoxide levels. Superoxide is necessary but not sufficient to kill cells. These results suggest that not all DNA damage is capable of inducing an increase in superoxide.

Chapter 4

Identification of a novel role for the targeting of *RAD1* in combination agent activities

Introduction

The model system *Saccharomyces cerevisiae* is a useful tool for assessing the effects of anticancer drugs in different genetic backgrounds (Simon and Bedalov, 2004). Here we utilize NER deficient strains that are lacking either *RAD1* or *RAD14* in the context of other DNA damage management deficiencies. Both *RAD1* and *RAD14* are essential for the function of NER, however *RAD1* mediates minor roles in REC and BER (Friedberg et al., 2006). We show here that in the context of certain DNA damage management deficient strains and under certain drug conditions, the *rad1* mutants are more sensitized to a variety of DNA damaging treatments, suggesting that Rad1 (or the human homolog XPF) would be a better target for a small molecule inhibitor than Rad14 (or the human homolog XPA) (Friedberg et al., 2006).

Experimental Procedures

Cytotoxicity assay to screen anticancer drugs. The cytotoxicity assay was performed as described in Chapter 2: Experimental Procedures with the following changes and additions. The strains used to compare the difference in sensitivities between Rad1 and Rad14 deficiencies are listed in Table 4.1. For experiments with UV irradiation, 15 mL of cells were placed in a 15-mm Petri dish and exposed to a range of UV-C doses. Cells were then placed in the dark to eliminate photoreactivation–mediated repair of

cyclobutane pyrimidine dimers and 1 ml aliquots were processed as above. Methyl methane sulfonate (MMS) was suspended in water for use in DNA damage cytotoxicity experiments.

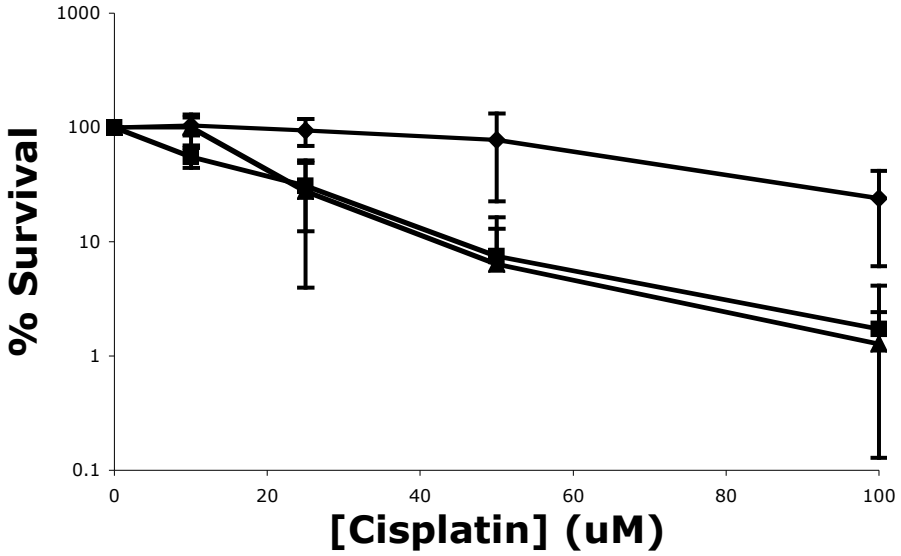
Table 4.1. Isogenic *S. cerevisiae* strains defective in NER (*Δrad1* or *Δrad14)* in different DNA damage management pathway backgrounds.

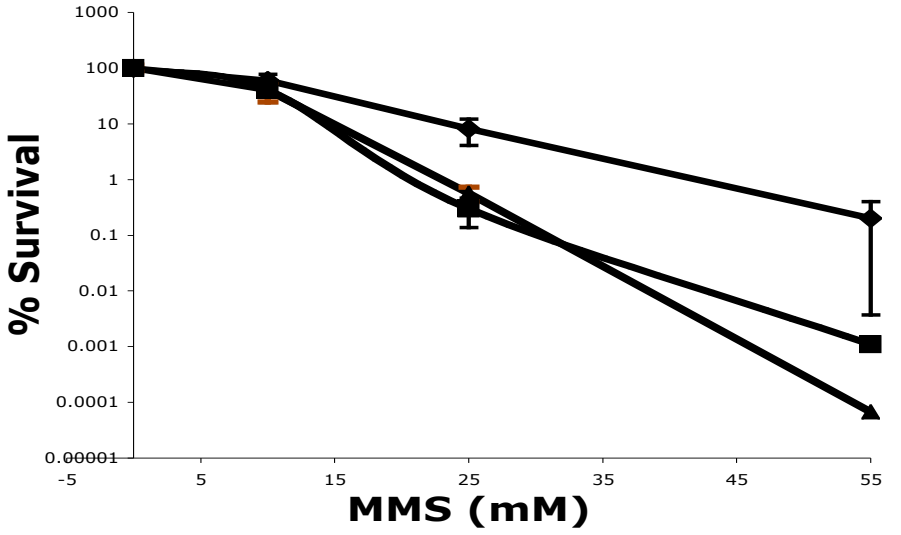
Strain	**Genotype**
Wildtype (WT)	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R*
NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG*
NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad14Δ::kanR*
BER/NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ1::HIS3 rad1Δ::hisG*
BER/NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R ntg1Δ::LEU2 ntg2Δ::hisG apn1Δ1::HIS3 rad14Δ::kanR*
NER/REC-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG rad52Δ::URA3*
NER/REC-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad14Δ::kanR rad52Δ::URA3*
TLS/NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad1Δ::hisG rev3Δ::kanR*
TLS/NER-defective	*MAT∝ ade2-101$_{oc}$ his3Δ200 ura3ΔNco lys2ΔBgl leu2-R rad14Δ::kanR rev3Δ::kanR*

Results

Sensitivities to cisplatin, methyl methane sulfonate (MMS), or UV-C of *Δrad1* and *Δrad14* in otherwise wildtype cells. We analyzed the sensitivity of the *Δrad1* and *Δrad14* strains when exposed to a variety of agents. MMS induces DNA damage that is repaired by BER (Friedberg et al., 2006). Cisplatin induces damage processed by NER,

REC and TLS (Beljanski et al., 2004). UV-C induced damage is processed by NER (Friedberg et al., 2006). Both *Δrad1* and *Δrad14* strains are equally sensitized to MMS, cisplatin and UV-C treatment as compared to wildtype cells (Figure 4.1).





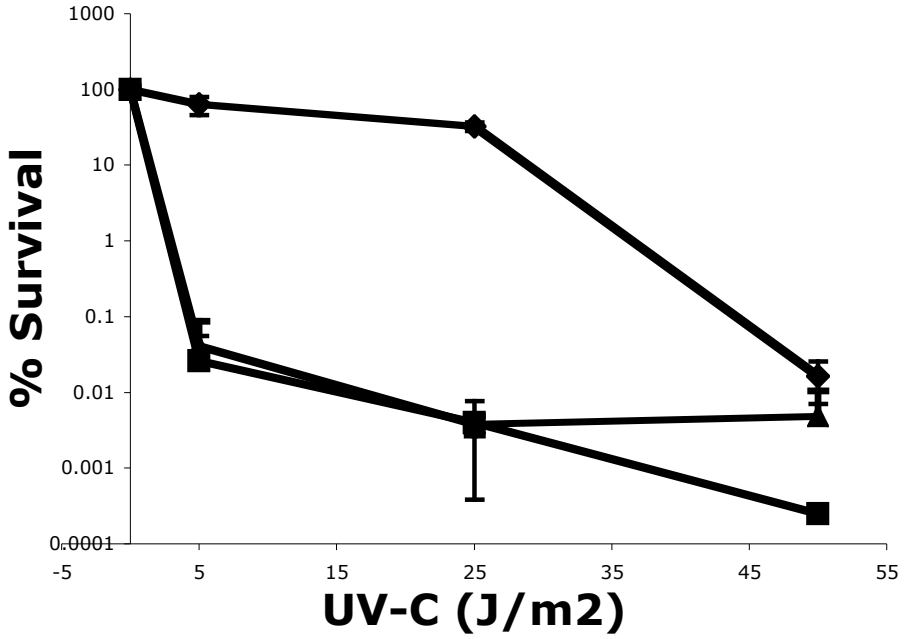
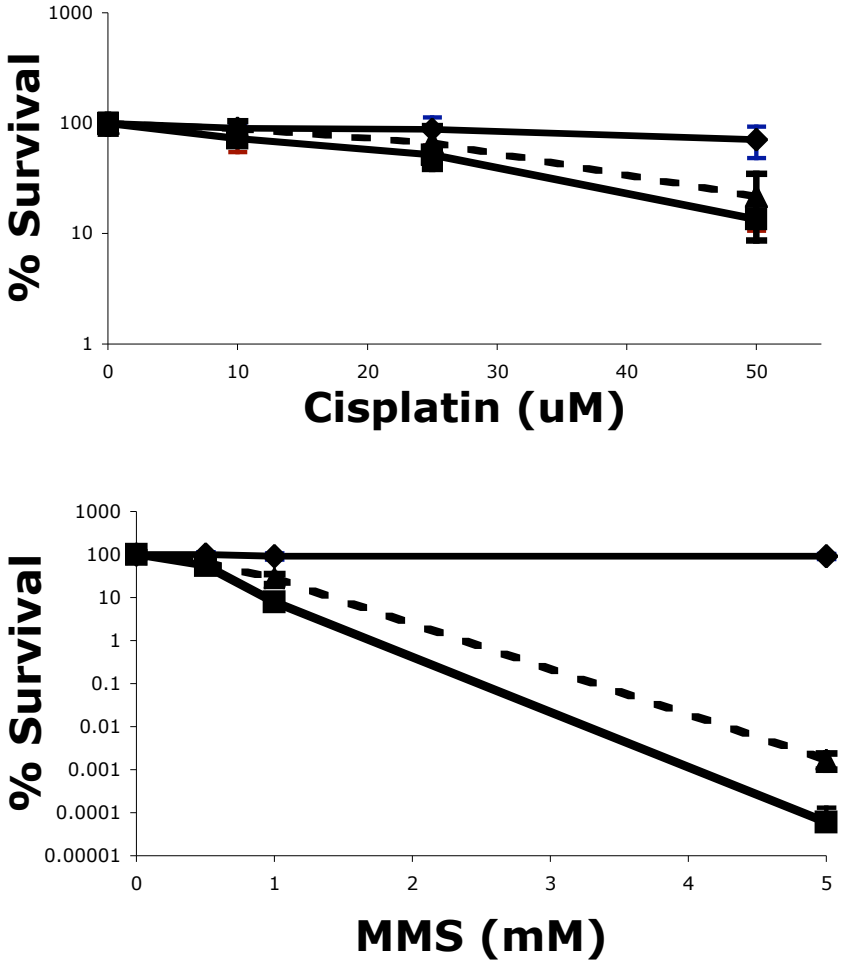


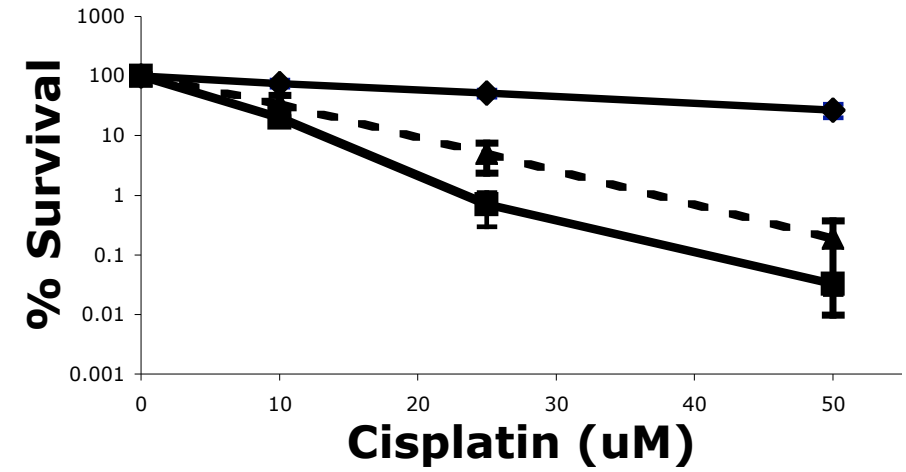
Figure 4.1. NER-deficient (*Δrad1* (■) and *Δrad14 (*▲*)*) strains are sensitized to cisplatin, MMS and UV-C and the wildtype strain is not (◆). Yeast cells were dosed with cisplatin or MMS or exposed to UV-C and survival and standard deviation calculated as described in methods.

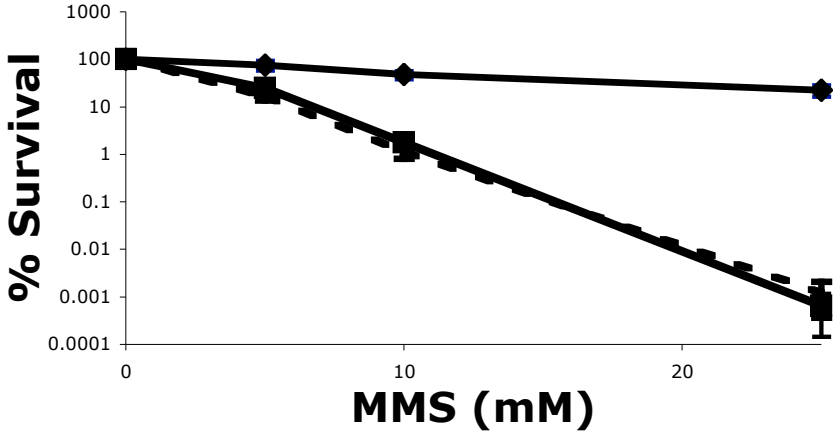
Sensitivities to cisplatin, methyl methane sulfonate (MMS), or UV-C of *Δrad1* and *Δrad14* in a DNA damage management deficient background. We decided to investigate the roles that Rad1 and Rad14 might have within the context of different repair capacities. Since Rad1 has minor functions in both REC and BER, we analyzed the NER-/REC- and BER-/NER- strains. The TLS-/NER- strains were also included. When treated with cisplatin, the TLS-/NER- (*Δrad1*) and NER-/REC- (*Δrad1*) strains were modestly but significantly more sensitized compared to the *Δrad14* strains (Figure 4.2 B & C). Only the BER-/NER- (*Δrad1*) strain was more sensitized to MMS than the corresponding *Δrad14* strain (Figure 4.2 A).

A


1000
100
10
1
% Survival
0
10
20
30
40
50
Cisplatin (uM)
1000
100
10
1
0.1
0.01
0.001
0.0001
0.00001
% Survival
0
1
2
3
4
5
MMS (mM)

B


1000
100
10
1
0.1
0.01
0.001
% Survival
0
10
20
30
40
50
Cisplatin (uM)


1000
100
10
1
0.1
0.01
0.001
0.0001
% Survival
0
10
20
MMS (mM)

C

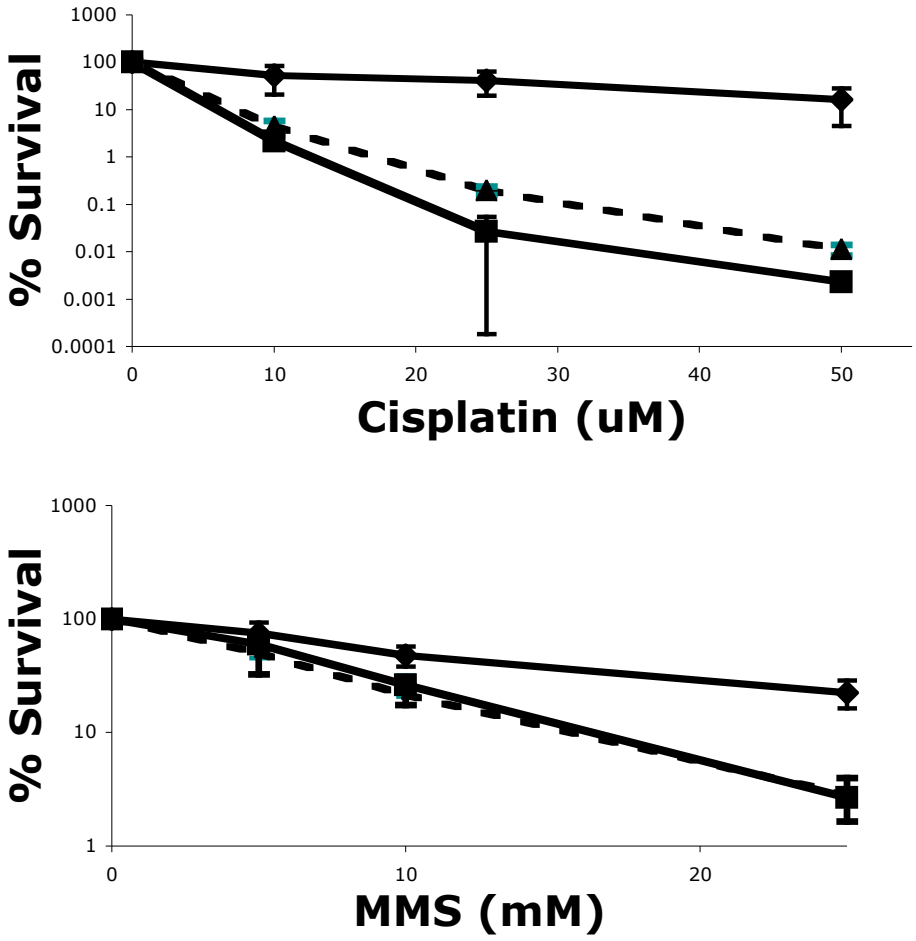


Figure 4.2. Strain sensitivities are influenced by DNA repair background, drug exposure, and *rad1* or *rad14* deletion. The strains were analyzed in groups as shown, (A) WT (◆), BER-/NER- (*Δrad1*) (■) and BER-/NER- (*Δrad14) (dashed* ▲), (B) WT (◆), NER-/REC- (*Δrad1*) (■) and NER-/REC- (*Δrad14*) *(dashed* ▲) and (C) WT (◆), TLS-/NER- (*Δrad1*) (■) and TLS-/NER- (*Δrad14*) *(dashed* ▲). Yeast cells were dosed with cisplatin or MMS and survival and standard deviation were determined as described in methods.

Discussion

Sensitivities to cisplatin, MMS, or UV-C of *Δrad1* and *Δrad14* in otherwise wildtype cells. There was no significant difference between the sensitivities of the *Δrad1* and *Δrad14* strains when exposed to cisplatin, MMS or UV-C. This result may be expected since Rad1 is thought to mediate relatively minor roles in BER and REC, which may only be revealed when these pathways are otherwise overwhelmed or compromised.

Sensitivities to cisplatin, methyl methane sulfonate (MMS), or UV-C of *Δrad1* and *Δrad14* in a DNA damage management system deficient background. Under some drug exposure conditions in certain DNA damage management deficient backgrounds, the *Δrad1* strains display greater sensitivity than the corresponding *Δrad14* strains. Addition of *Δrad1* to REC- and TLS- strains sensitized the mutants to cisplatin as compared to the corresponding *Δrad14* strains. However, the *Δrad1* BER-/NER- strain was more sensitized to MMS exposure compared to the corresponding *Δrad14* strain. The roles of Rad1 in the DNA damage management pathways other than NER appear to be minimal, due to the slight sensitivities of the *Δrad1* strain in repair deficient backgrounds, at least within the context of the types of DNA damaging agents used in the studies. As previously shown (Figure 2.1 and (Beljanski et al., 2004)), deficiencies in multiple DNA damage management pathways result in exquisite sensitivity to DNA damaging anticancer agents. Rad1 would be an excellent choice for a target of a small molecule inhibitor to be used in combination with other anticancer agents or in certain DNA damage management capacities backgrounds.

Conclusions

Under certain drug exposure conditions and DNA damage management pathway deficiencies, the *Δrad1* strains are more sensitized than the corresponding *Δrad14* strains. The role of Rad1 in DNA damage management pathways other than NER is minimal. If developing a small molecule inhibitor to NER, Rad1 would be a better choice due to the targeting of multiple pathways with one agent.

Chapter 5

Conclusions

S. cerevisiae as a model system for screening anticancer agents. Here we have employed isogenic strains of *Saccharomyces cerevisiae* with various DNA damage management system capacities. In addition to the traditional advantages of using yeast including fast generation times and ease of genetic manipulation, we utilized the ability to generate isogenic strains and strains lacking mitochondrial DNA for our studies. This model system has proven useful for identifying the DNA damage management pathways that are involved in processing various types of DNA damage (Chapter 2 and (Beljanski *et al*., 2004). The isogenic panels have also been used here to determine the difference between the sensitivity of *Δrad1* and *Δrad14* in various DNA repair backgrounds (Chapter 4).

Cellular response to cisplatin. Using the isogenic panel of DNA damage management deficient strains, we have determined that TLS, REC and NER are the major pathways responsible for processing the DNA damage induced by cisplatin treatment (Chapter 2 and (Beljanski *et al*., 2004)). When treated with cisplatin, superoxide levels increase in a dose-dependent manner in cells regardless of the DNA damage management capacity. This superoxide increase occurs in the mitochondria and throughout the cell. The superoxide level increase in response to cisplatin is not due to DNA damage to the mitochondria. In addition, if DNA damage to nuclear DNA results in an increase in superoxide, it is due to the DNA monoadducts that both cisplatin and transplatin induce. However, the complex bifunctional DNA adducts that cisplatin forms could cause the cytotoxicity that makes cisplatin a successful anticancer agent (Chapter 3).

References

Alexander RW (1995) Hypertension and the pathogenesis of atherosclerosis: oxidative stress and the mediation of arterial inflammatory response: a new perspective. *Hypertension* **25**(2):155-161.

Babior BM (1984) The respiratory burst of phagocytes. *J Clin Invest* **73**(3):599-601.

Beckman KB and Ames BN (1997) Oxidative decay of DNA. *J Biol Chem* **272**:19633-19636.

Beljanski V, Marzilli LG and Doetsch PW (2004) DNA Damage-Processing Pathways Involved in the Eukaryotic Cellular Response to Anticancer DNA Cross-Linking Drugs. *Molecular Pharmacology* **65**(6):1496-1506.

Boudvillain M, Dalbies R, Aussourd C and Leng M (1995) Intrastrand cross-links are not formed in the reaction between transplatin and native DNA: relation with clincal inefficiency of transplatin. *Nucleic Acids Research* **23**(13):2381-2388.

Boulikas T and Vougiouka M (2004) Recent clinical trials using cisplatin, carboplatin and their combination chemotherapy drugs. *Oncology Reports* **11**:559-595.

Brand MD (1990) The proton leak across the mitochondrial inner membrane. *Biochimica et Biophysica Acta* **1018**:128-133.

Cooke MS, Evans MD, Dizdaroglu M and Lunec J (2003) Oxidative DNA damage: mechanisms, mutation, and disease. *FASEB J* **17**:1195-1214.

Costa V and Moradas-Ferreira P (2001) Oxidative stress and signal transduction in Saccharomyces cerevisiae: insights into ageing, apoptosis and diseases. *Mol Aspects Med* **22**(4-5):217-246.

Dreher D and Junod A (1996) *European Journal of Cancer* **32**(1):30-38.

Evert BA, Salmon TB, Song B, Jingjing L, Siede W and Doetsch PW (2004) Spontaneous DNA Damage in Saccharomyces cerevisiae Elicits Phenotypic Properties Similar to Cancer Cells. *The Journal of Biological Chemistry* **279**(21):22585-22594.

Fahl WE, Lalwani ND, Watanabe T, Goel SK and Reddy JK (1984) DNA damage related to increased hydrogen-eroxide generation by hypolipidemic drug-indcued liver peroxisomse. *Proc Natl Acad Sci* **81**:7827-7830.

Ferry KV, Hamilton TC and Johnson SW (2000) Increased Nucleotide Excision Repair in Cisplatin-Resistant Ovarian Cancer Cells. *Biochemical Pharmacology* **60**:1305-1313.

Finkel T (1998) Oxygen radicals and signaling. *Current Opinion in Cell Biology* **10**:248-253.

Finkel T and Holbrook NJ (2002) Oxidants, oxidative stress and the biology of ageing. *Nature* **408**(6809):239-247.

Friedberg EC, Walker GC, Siede W, Wood RD, Schultz RA and Ellenberger T (2006) *DNA Repair and Mutagenesis*. ASM Press, Washington D.C.

Fruehauf JP and Frank L. Meyskens J (2007) Reactive Oxygen Species: A Breath of Life or Death? *Clin Cancer Res* **13**(3):789-794.

Hanahan D and Weinberg RA (2000) The Hallmarks of Cancer. *Cell* **100**:57-70.

Harman D (1981) *Proc Natl Acad Sci* **78**(11):7124-7128.

Karihtala P and Soini Y (2007) Reactive oxygen species and antioxidant mechanisms in human tissues and their relation to malignancies. *APMIS* **115**:81-103.

Kelland L (2007) The resurgence of platinum-based cancer chemotherapy. *Nat Rev Cancer* **7**(8):573-584.

Kopnin PB, Agapova LS, Kopnin BP and Chumakov PM (2007) Repression of Sestrin Family Genes Contributes to Oncogenic Ras-Induced Reactive Oxygen Species Up-regulation and Genetic Instability. *Cancer Research* **67**(10):4671-4678.

Krogh BO and Symington LS (2004) Recombination Proteins in Yeast. *Annual Rev Genet* **38**:233-271.

Kubicek S, O'Sullivan RJ, August EM, Hickey ER, Zhang Q, Teodoro ML, Rea S, Mechtler K, Kowalski JA, Homon CA, Kelly TA and Jenuwein T (2007) Reversal of H3K9me2 by a Small-Molecule Inhibitor for the G9a Histone Methyltransferase. *Molecular Cell* **25**:473-481.

Kuo MT and Savaraj N (2006) Roles of Reactive Oxygen Species in Hepatocarcinogenesis and Drug Resistance Gene Expression in Liver Cancers. *Molecular Carcinogenesis* **45**:701-709.

Kuzminov A (2001) DNA replication meets genetic exchange: Chromosomal damage and its repair by homologous recombination. *PNAS* **98**(15):8461-8468.

Lambeth JD (2004) Nox enzymes and the biology of reactive oxygen. *Nature Reviews Immunology* **4**:181-189.

Lengauer C, Kinzler KW and Vogelstein B (1998) Genetic instabilities in human cancers. *Nature* **396**:643-649.

Lin X, Trang J, Okuda T and Howell SB (2006) DNA Polymerase Zeta Accounts for the Reduced Cytotoxicity and Enhanced Mutagenicity of Cisplatin in Human Colon Carcinoma Cells That Have Lost DNA Mismatch Repair. *Cancer Therapy: Preclinical*.

Luo J, Solimini NL and Elledge SJ (2009) Principles of Cance Therapy: Oncogene and Non-oncogene Addiction. *Cell* **136**:823-837.

Malins DC, Polissar NL and Gunselman SJ (1996) Progression of human breast cancers to the metastatic state is linked to hydroxyl radical-induced DNA damage. *Proc Natl Acad Sci* **93**:2557-2563.

McGurk CJ, Cummings M, Koberle B, Hartley JA, Oliver RT and Masters JR (2006) Regulation of DNA Repair Gene Expression in Human Cancer Cell Lines. *Journal of Cellular Biochemistry* **97**:1121-1136.

Morrison AJ and Shen X (2009) Chromatin remodeling beyod transcription: the INO80 and SWR1 complexes. *Molecular Cell Biology* **10**:373-383.

Nicholls DG (18974) The Influence of Respiration and ATP Hydrolysis on the Proton-Electrochemical Gradient across the Inner Membrane of Rat-Liver Mitochondria as Determined by Ion Distribution. *European Journal of Biochemistry* **50**(1):305-315.

Pelicano H, Carney D and Huang P (2004) ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates* **7**:97-110.

Rowe LA, Degtyareva N and Doetsch PW (2008) DNA damage- induced reactive oxygen species (ROS) stress response in Saccharomyces cerevisiae. *Free Radical Biology & Medicine* **45**:1167-1177.

Salmon TB, Evert BA, Song B and Doetsch PW (2004) Biological consequences of oxidative stress-induced DNA damage in Saccharomyces cerevisiae. *Nucleic Acids Research* **32**(12):3712-3723.

Salvador A, Sousa J and Pinto RE (2001) Hydroperoxyl, superoxide and pH gradients in the mitochondrial matrix: A theoretical assessment. *Free Radical Biology & Medicine* **31**:1208-1215.

Saxowsky TT, Meadows KL, Klungland A and Doetsch PW (2008) 8-Oxoguanine-mediated transcriptional mutagenesis causes Ras activation in mammalian cells. *PNAS* **105**(48):18877-18882.

Siddik ZH (2003) Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22**:7265-7279.

Simon JA and Bedalov A (2004) Yeast as model system for anticancer drug discovery. *Nat Rev Cancer* **4**:1-8.

Siomek A, Tujakowski J, Gackowski D, Rozalski R, Foksinski M, Dziaman T, Roszkowski K and Olinski R (2006) Severe oxidatively damaged DNA after cisplatin treatment of cancer patients. *Int J Cancer* **119**:2228-2230.

Swanson RL, Morey NJ, Doetsch PW and Jinks-Robertson S (1999) Overlapping Specificities of Base Excision Repair, Nucleotide Excision Repair, Recombination, and Translesion Syntesis Pathways for DNA base Damage in Saccharomyces cerevisiae. *Molecular and Cellular Biology* **19**(4):2929-2935.

Szatrowski TP and Nathan CF (1991) Production of Large Amounts of Hyrdogen Peroxide by Human Tumor Cells. *Cancer Research* **51**:794-798.

Vafa O, Wade M, Kern S, Beeche M, Pandita TK, Hampton GM and Wahl GM (2002) c-Myc Can Induce DNA Damage, Increase Reactive Oxygen Species, and Mitigate p53 Function: A Mechanism for Oncogene-Induced Genetic Instability. *Molecular Cell* **9**:1031-1044.

Wheeler MD (2003) Endotoxin and Kupffer cell activation in alcoholic liver disease. *Alcohol Res Health* **27**:300-306.

Wilborn F and Brendel M (1989) Formation and stability of interstrand cross-links induced by cis- and trans-diamminedichloroplatinum (II) in the DNA of Saccharomyces cerevisiae strains differing in repair capacity. *Current Genetics* **16**:331-338.

Wu F, Lin X, Okuda T and Howell SB (2004) DNA Polymerase Zeta Regulates Cisplatin Cytotoxicity, Mutagenicity, and The Rate of Development of Cisplatin Resistance. *Cancer Research* **64**:8029-8035.