The Anticancer Ruthenium Complex KP1019 Induces DNA Damage, Leading to Cell Cycle Delay and Cell Death in Saccharomyces cerevisiae

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ABSTRACT

The anticancer ruthenium complex trans-\{(tetrachlorobis(1H-indazole))ruthenate(III)\}, otherwise known as KP1019, has previously been shown to inhibit proliferation of ovarian tumor cells, induce DNA damage and apoptosis in colon carcinoma cells, and reduce tumor size in animal models. Notably, no dose-limiting toxicity was observed in a Phase I clinical trial. Despite these successes, KP1019’s precise mechanism of action remains poorly understood. To determine whether Saccharomyces cerevisiae might serve as an effective model for characterizing the cellular response to KP1019, we first confirmed that this drug is internalized by yeast and induces mutations, cell cycle delay, and cell death. We next examined KP1019 sensitivity of strains defective in DNA repair, ultimately showing that rad1Δ, rev3Δ, and rad52Δ yeast are hypersensitive to KP1019, suggesting that nucleotide excision repair (NER), translesion synthesis (TLS), and recombination each play a role in drug tolerance. These data are consistent with published work showing that KP1019 causes interstrand cross-links and bulky DNA adducts in mammalian cell lines. Published research also showed that mammalian cell lines resistant to other chemotherapeutic agents exhibit only modest resistance, and sometimes hypersensitivity, to KP1019. Here we report similar findings for S. cerevisiae. Whereas gain-of-function mutations in the transcription activator-encoding gene PDR1 are known to increase expression of drug pumps, causing resistance to structurally diverse toxins, we now demonstrate that KP1019 retains its potency against yeast carrying the hypermorphic alleles PDR1-11 or PDR1-3. Combined, these data suggest that S. cerevisiae could serve as an effective model system for identifying evolutionarily conserved modulators of KP1019 sensitivity.

Introduction

Platinum-based chemotherapeutics are often used to treat solid tumors, including testicular and ovarian carcinomas (recently reviewed by Kelland, 2007). However, the renal, neurologic, gastroenterologic, and marrow toxicity of cisplatin and related drugs have led researchers to develop other metal complexes in an effort to maximize efficacy while minimizing negative side effects (Clarke et al., 1999; Allardyce and Dyson, 2001; Zhang and Lippard, 2003). One such promising chemotherapeutic agent is the anticancer ruthenium complex trans-\{(tetrachlorobis(1H-indazole))ruthenate(III)\}, also known as KP1019 (structure available in Hartinger et al., 2008). This drug has been shown to reduce tumor size in rats (Berger et al., 1989) and trigger apoptosis in vitro (Kapitza et al., 2005). Furthermore, in early clinical trials, no dose-limiting toxicity was found, and five out of six patients either stabilized or improved (Hartinger et al., 2008; Lentz et al., 2009). Preliminary studies also suggest that KP1019 might be promising in the treatment of drug-resistant tumors, as it remains effective against cancer cell lines that are highly resistant to other chemotherapeutic agents. Specifically, cell
Despite KP1019's successes to date, its mechanism of action remains a matter of debate. For example, KP1019 has been shown to bind to transferrin, and transferrin receptor-mediated endocytosis has been implicated as a mechanism for drug internalization (Kratz et al., 1994; Pongratz et al., 2004). However, drug uptake occurs in the absence of KP1019-loaded transferrin (Pongratz et al., 2004), suggesting that other modes of drug internalization exist. Furthermore, even when the transferrin pathway is involved in KP1019 endocytosis, the mechanism by which the drug exits endosomes and gains access to intracellular targets remains unclear.

In addition to questions about KP1019 transport, several issues remain unresolved with respect to KP1019's intracellular mechanism of action. For example, KP1019 treatment triggered hydrogen peroxide production in colorectal cancer cell lines, and the resulting oxidative stress caused DNA damage and apoptosis (Kapitza et al., 2005). Furthermore KP1019 toxicity was largely ablated by addition of antioxidants, again suggesting that DNA damage is a secondary effect of the drug. In contrast, Frühauf and Zeller (1991) showed that KP1019 forms protein-DNA and interstrand cross-links, indicating that the drug may damage DNA directly. Moreover, biochemical DNA relaxation assays have demonstrated that KP1019 acts as a topoisomerase II poison in vitro, ultimately forming a drug-containing cleavage complex (Vashisht Gopal and Kondapi, 2001).

In addition to the controversy regarding the mechanism by which KP1019 damages DNA, non-nuclear targets have been implicated by the observation that KP1019 is found pre-actively to the effects of KP1019 (Heffeter et al., 2005).

In vitro, ultimately forming a drug-containing cleavage complex (Vashisht Gopal and Kondapi, 2001).

Here we show that KP1019 inhibits yeast growth through both cytotoxic and cytostatic effects. Furthermore, our findings are consistent with previous studies indicating that KP1019 damages DNA either directly or indirectly, as this drug is capable of inducing mutations and recombination in yeast.

### Materials and Methods

**Yeast Strains and Growth Conditions.** Yeast strains used in this study are listed in Table 1. Yeast were grown under standard conditions, at 30°C using rich medium yeast extract peptone dextrose (YPD) (1% yeast extract, 2% Bacto-peptone, 2% dextrose) or synthetic complete media (SDC) as indicated (Sherman et al., 1986). For experiments involving plasmids, a standard lithium acetate transformation protocol (Gietz and Woods, 2002) was used, and transformants were selected on minimal media lacking the component necessary for plasmid maintenance. RAD52 deletion strains unique to

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Strains used in this study</th>
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<tr>
<td>Strain</td>
<td>Genotype</td>
</tr>
<tr>
<td>SJR0751</td>
<td>MATa ade2-101, His3Δ200</td>
</tr>
<tr>
<td></td>
<td>ura3ΔNco lys2ΔBgl leu2-R</td>
</tr>
<tr>
<td>SJR0897</td>
<td>MATa ade2-101, His3Δ200</td>
</tr>
<tr>
<td></td>
<td>ura3ΔNco lys2Δ3'</td>
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<td></td>
<td>URA3 lys2ΔBgl leu2-R</td>
</tr>
<tr>
<td></td>
<td>HTB2-mCherry-SpHIS5</td>
</tr>
<tr>
<td>JB206-1C</td>
<td>MATa ade2 WHIS-GFP-kanMX</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>BY4742</td>
<td>MATa his3Δ1 leu2Δ30 lys2Δ30</td>
</tr>
<tr>
<td>moh2Δ30</td>
<td>In BY4742</td>
</tr>
<tr>
<td>Rad52-GFP</td>
<td>MATa his3Δ1 leu2Δ30 met15Δ30</td>
</tr>
<tr>
<td>DSCO025</td>
<td>MATa ade2-101, His3Δ200</td>
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<tr>
<td></td>
<td>ura3ΔNco lys2ΔBgl leu2-R</td>
</tr>
<tr>
<td>DSCO035</td>
<td>ndt1Δ:LEU2 ndt2::hisG apn1::HIS3 in DSCO025</td>
</tr>
<tr>
<td>DSCO036</td>
<td>rad1::hisG in DSCO025</td>
</tr>
<tr>
<td>DSCO133</td>
<td>rad2::KANΔ in DSCO025</td>
</tr>
<tr>
<td>PHY528</td>
<td>rad52::KANΔ in DSCO025</td>
</tr>
<tr>
<td>PHY532</td>
<td>rad52::KANΔ in DSCO036 (rad1::hisG)</td>
</tr>
<tr>
<td>LKY118</td>
<td>MATa can1-100 ade2-1 HIS3- leu2-3,</td>
</tr>
<tr>
<td></td>
<td>112 trp1-1 ura3-1 lys2</td>
</tr>
<tr>
<td></td>
<td>MATa PDR1-11 ade2-1 HIS3- leu2-3,</td>
</tr>
<tr>
<td></td>
<td>112 trp1-1 ura3-1 lys2</td>
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<tr>
<td>IL125-2B</td>
<td>MATa PDR1 his1</td>
</tr>
<tr>
<td>USS0-18C</td>
<td>MATa PDR1-3 his1</td>
</tr>
</tbody>
</table>
this study were generated by standard methods. Briefly, the rad52:: kanMX cassette was PCR amplified from a commercially available rad52 null strain (Winzeler et al., 1999; Open Biosystems; Lafayette, CO). The amplified deletion cassette was transformed into wild-type (DSC0025) or rad11 (DSC0036) yeast using lithium acetate as previously described. Putative deletion strains were isolated in YPD + 200 μg/ml G418. Genotypes were confirmed by PCR.

**Drug Synthesis.** KP1019 was synthesized using a protocol adapted from Lipsoner et al. (1996). Briefly, 1g RuCl3·3H2O was added to 20 ml of 12 M HCl and 20 ml of ethanol. The solution was then refluxed for 1 hour and cooled to 40°C. The ethanol was then removed from the solution through the use of a rotary evaporator. Next, 12 M HCl was added to a final volume of 40 ml, and 1.87 g of indazole was heated to 70°C in 30 ml 12 M HCl, then 20 ml of the Ru (III) solution was added to the hot indazole solution. This mixture was heated for 15 minutes at 80–90°C. The new solution was cooled to room temperature with stirring. The resulting solid was washed by collecting filtration, and then stirred in approximately 75 ml of H2O at room temperature for 2 hours. The solid was filtered and subsequently washed with cold ethanol followed by cold diethyl ether.

The drug was dried under vacuum for 18–24 hours. KP1019 purity was verified by UV-visible spectroscopy, elemental analysis and determination of reduction potential. When dissolved in synthetic yeast medium, KP1019 was found to be stable for at least 48 hours as determined by lack of change in the drug's UV-visible absorption spectrum (data not shown).

**Survival, Mutation, and Recombination Assay.** Rates of yeast survival, frequency of mutation, and frequency of recombination were determined using standard methods (Jinks-Robertson and Petes, 1993). Briefly, yeast were cultured in YPD to a final optical density at 600 nm (OD600) of 0.5 to 0.6. Cells were then washed with water, and approximately one OD equivalent of cells was incubated with each of the indicated concentrations of drug dissolved in water for 30 minutes at 30°C. Following drug treatment, cells were washed three times with water and serial diluted to obtain approximately 50–200 colonies per plate when transferred to YPD to determine percent survival or to selective media to identify mutants or recombinants. The S. cerevisiae strain JBY0751 was used for survival and mutation assay, and can1 mutants were identified by plating on SDC minus arginine but containing 60 μg/ml canavanine. The S. cerevisiae strain SJR0897 was used for analysis of drug-induced recombination; p++ recombinants were identified by plating on SDC minus lysine.

**KP1019 Uptake.** Wild-type yeast (BY4742) were grown in SDC until mid-log phase (OD600 approximately 0.8). This culture was then split into four 20 ml aliquots that were each diluted 2-fold with fresh media either lacking or containing KP1019 (20 μg/ml) final concentration. One of the resulting 40 ml KP1019-containing samples was pelleted immediately, washed, and ashed as described later in this section. The two remaining KP1019-containing samples and the 0 μg/ml KP1019 control were incubated for 2 hours at 30°C with shaking.

The culture density of each sample was then measured, cells were washed four times with water, and cell pellets were stored at −20°C. This culture-splitting, treatment, and cell-washing protocol was repeated for 48 hours. Cooled ashes were dissolved in 1.8 ml of nitric acid and 3.2 ml of deionized water. Dissolved ashes were analyzed by atomic absorption spectroscopy using a PerkinElmer AAnalyt 800 (Waltham, MA) with a graphite furnace and a ruthenium lamp. Each biologic sample was analyzed in triplicate and compared with a standard curve (0–99 ppb), samples for which were also read three times each.

**Drug Sensitivity.** Drug-containing SDC was subjected to 2-fold serial dilution across the wells of a microtiter plate. Overnight cultures of yeast were diluted to OD600 = 0.1 and subsequently diluted 20-fold more in SDC. An equal volume of diluted cell suspension was added to each well of the microtiter plate. After an 18- to 24-hour incubation at 30°C, the growth of each strain at each concentration of drug was recorded as absorbance at 630 nm using a BioTek (Winooski, VT) microtiter plate reader.

**Cell Cycle Analysis.** The S. cerevisiae strain JBY206-1C was grown to early log phase (OD600 of 0.25–0.3), split, and half was treated with varying amounts of KP1019 (0, 20, 40, and 60 μg/ml). Cultures were harvested for microscopic image analysis. Samples for image analysis were removed from treated and untreated cultures, sonicated for 10 seconds at 4°C output on Sonicator Dismembrator Model 100 (Thermo Fisher Scientific, Hampton, NH) to disrupt cellular clumps, and placed directly on a microscope slide for image capture. Images were captured using a 20× objective with the LSM710 Confocal Imaging System (Carl Zeiss AG, Oberkochen, Germany) with ZEN software (Carl Zeiss AG, Oberkochen, Germany). Differential interference contrast imaging and fluorescent microscopy images were captured to allow visualization of cellular morphology and nuclear morphology respectively. Cellular morphology was scored based on the presence or absence of a bud. Nuclear morphology was scored based on number, shape, and position of nuclei as defined by histone H2-mCherry signal. Between 500 and 1000 cells were scored for the cellular and nuclear morphology of each drug dose and time point. Three independent time courses were carried out for each treatment dosage. Images that were scored for cellular and nuclear morphology were also used to make cell area measurements. Cell areas were measured using ImageJ image analysis software (National Institutes of Health, Bethesda, MD). To measure changes in cell size of the budding cell population specifically, the threshold for area measurement was set between 30 and 80 μm² for all images to exclude unbudded cells as well as large artifacts. A minimum of 500 cells was counted for each sample, with several samples exceeding 1000 counted cells. Measurements were made from three independent time courses for each treatment dosage. The same images were analyzed both for cell size and for cellular and nuclear morphology.

**β-Galactosidase Assay.** The genotoxicity of KP1019 was established using wild-type yeast strain BY4742 transformed with the pZZ2-RNR3-lacZ reporter construct (Zhou and Elledge, 1992). Specifically, transformed cells were cultured to mid-log phase (OD600 0.5–1.0) in selective media then treated with KP1019 (0, 20, 40, 60, 80, or 100 μg/ml) dissolved in the same type of medium. Samples were incubated for 3 hours to allow for gene induction, at which point beta-galactosidase activity was measured using the permeabilized cell assay described by Guarente (1983). Fold induction was determined by dividing each sample's β-galactosidase activity by the activity measured for the 0 μg/ml KP1019 control.

**Analysis of Rad52-GFP Localization.** Overnight cultures of Rad52-GFP yeast were subcultured to mid-log phase (OD600 0.5–1.0) in SDC at 23°C. KP1019 was dissolved in SDC at a concentration of 1 mg/ml and then added to the yeast to achieve the desired final concentration. For no drug controls, the same volume of SDC was added to an aliquot of yeast culture. Cells were incubated with KP1019 at 23°C for 1 hour. Approximately 20 minutes prior to visualization, 4,6-diamidino-2-phenylindole (DAPI) was added to a final concentration of 1 μg/ml. GFP and DAPI were visualized using the 100× objective of a Zeiss Axiophot 2 plus; images were captured with AxioVision software. For cell cycle dependence of Rad52-GFP focus formation, cellular morphology was scored based on the presence and size of a bud.

**Results**

**KP1019 Is Internalized by and Kills Yeast.** Previous studies have demonstrated that the promising anticancer ruthenium complex KP1019 triggers apoptosis of colorectal cancer cells in vitro (Kapitza et al., 2005). Likewise, we...
observed a dose-dependent increase in cell death when the budding yeast *S. cerevisiae* was treated with KP1019 (Fig. 1A). Given that KP1019 has been reported to be internalized by the transferrin pathway (Kratz et al., 1994; Pongratz et al., 2004) and given that *S. cerevisiae* does not use this pathway for iron uptake, we wanted to verify that KP1019 was, in fact, being internalized by yeast. As seen in Fig. 1B, a dramatic increase in cell-associated ruthenium was observed when yeast cells were incubated with 20μg/ml KP1019 for 2 hours. There was only a small difference between the amount of cell-associated ruthenium in the untreated control and the cells that were treated momentarily (0 min) with KP1019, suggesting that adherence to the cell wall is not likely to be a confounding variable.

**KP1019 Causes Cell Cycle Delay in *S. cerevisiae***. To determine whether cell cycle delay might explain the relative lack of cell death (Fig. 1) following treatment with concentrations of KP1019 that induce robust growth inhibition (Fig. 4, for example), we examined the impact of KP1019 on budding index. As seen in Fig. 2, KP1019 treatment led to the accumulation of large budded cells with elongated or partially separated nuclei that are located near or span the bud neck. Within 1 hour, all of the KP1019-treated samples were

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**Fig. 1.** KP1019 kills yeast. (A) as described under Materials and Methods, wild-type yeast (SJR0751) were treated with the indicated concentrations of KP1019 for 2 hours prior to washing with water and plating on rich media to assess survival (colony-forming units). Error bars represent the standard error of five trials. (B) wild-type (BY4742) yeast were treated with the indicated concentrations of KP1019 for 0 or 2 hours prior to washing, ashing, and analysis by atomic absorption spectroscopy. Error bars represent the standard error of three independent experiments, each of which included two replicates of the 2-hour treatment with 20 μg/ml KP1019. Asterisks indicate statistically significant differences in ruthenium uptake relative to the 0 μg/ml control. *P* < 0.05, **P** < 0.01.

**Fig. 2.** KP1019 triggers cell cycle delay. Yeast were treated with KP1019 and imaged as described under Materials and Methods. (A) representative microscopy showing accumulation of cells showing nuclei spanning and/or near bud neck. Size bar = 10 μm. (B) one representative trial showing accumulation of cells with nuclei spanning and/or near bud neck. At least 300 cells were scored for each time point at each KP1019 concentration. (C) average cell size for 0 drug (gray) and 60 μg/ml drug (black). Error bars represent 2× S.E. of three experimental trials. Asterisk indicates statistically significant difference in cell size relative to the 0 μg/ml control. *P* < 0.05.
significantly different from the no-drug control \((P < 0.05)\). By 3 hours, the dose dependence of this effect was apparent and statistically significant \((P < 0.05)\). Accumulation of this morphology suggests that the drug induced a delay in cell cycle progression during the anaphase stage of mitosis (Yang et al., 1997). This delay was dose dependent and occurred within the first cycle of cell division. Furthermore, KP1019-treated cells continued to grow during this cell cycle delay, as evidenced by an increase in the size of cells treated with KP1019 as compared with untreated cells (Fig. 2C). These data suggest that in addition to KP1019’s cytotoxicity, the drug’s impact on the cell cycle may play a major role in its ability to inhibit yeast growth.

**KP1019 Is Genotoxic in S. cerevisiae.** Previous studies in mammalian systems suggest that KP1019 damages DNA (Frühauf and Zeller, 1991; Kapitza et al., 2005). Therefore, we wanted to determine whether KP1019 is genotoxic in yeast, as drug-induced DNA damage could explain the drug’s impact on the cell cycle and survival. Induction of the reporter construct \(RNR3\)-\(lacZ\) can be used to identify agents that directly damage DNA and/or perturb DNA replication (Jia et al., 2002). As seen in Fig. 3A, KP1019 treatment induces \(RNR3\)-\(lacZ\) expression in a dose-dependent fashion. This increase in expression peaks at 40 \(\mu\)g/ml and subsequently tapers off, an effect that may be due to the cell death seen at higher doses of KP1019. To better characterize the type of genotoxicity caused by KP1019, we next examined the drug’s ability to cause loss-of-function mutations in the arginine transporter—encoding gene \(CAN1\) (Whelan et al., 1979). Fig. 3B shows that KP1019 is mutagenic in yeast, as elevating the concentration of KP1019 increases the frequency of colonies resistant to the toxic arginine analog canavanine. In addition to studying basic frequencies of drug-induced mutation, we used the split lysine assay to quantify frequencies of recombination (Jinks-Robertson and Petes, 1993). This experimental approach relies on a strain of yeast that carries two copies of the \(LYS2\) gene. One copy carries a loss-of-function mutation at the 3’ end whereas the other carries a loss-of-function mutation at the 5’ end. Thus, this yeast strain will remain auxotrophic for lysine unless recombination occurs between the two \(lys2\) alleles, resulting in a functional copy of the gene that restores lysine prototrophy. As seen in Fig. 3C, KP1019 induces recombination in a dose-dependent manner, suggesting that recombination may be an important mechanism by which cells cope with drug-induced DNA damage.

**RAD52 Influences S. cerevisiae Tolerance of KP1019.** To further examine the role of recombination in the cellular response to KP1019, we compared the KP1019 sensitivity of wild-type yeast and yeast that were recombination-deficient due to lack of \(RAD52\). As seen in Fig. 4A, deletion of \(RAD52\) dramatically increases KP1019 sensitivity. Specifically, the half-maximal inhibitory concentration \((IC_{50})\) for the wild-type strain is 5.0 \(\mu\)g/ml, whereas the \(IC_{50}\) for the \(rad52\) strain is 0.53 \(\mu\)g/ml. In addition to studying recombination-deficient yeast, we also examined the localization of Rad52-GFP. Prior research has shown that double-strand breaks trigger formation of Rad52-GFP foci within the nuclei of yeast cells (Lisby et al., 2001). Fig. 4C shows that KP1019 induces Rad52-GFP focus formation. Furthermore, the Rad52-GFP foci seem to be more prevalent in cells with buds (Fig. 4D). These data are consistent with the result that KP1019 induces recombination because, in the haploid Rad52-GFP strain, homologous copies of genes will only be present during and following S phase. It is important to note that no significant cell death, as indicated by methylene blue or propidium iodide staining, occurred within the time course of

**Fig. 3.** KP1019 is genotoxic. (A) exponentially growing yeast transformed with the \(RNR3\)-\(lacZ\) reporter construct were treated with the indicated concentrations of KP1019 for 3 hours before measuring \(\beta\)-galactosidase activity. (B) the frequency of KP1019-induced mutation was measured by treating wild-type strain SJR0751 with KP1019 for 2 hours. Cells were subsequently washed and plated on YPD to determine percent survival and on selective media to identify \(CANr\) mutants. (C) the frequency of KP1019-induced recombination was measured by treating SJR0897 with KP1019 for 2 hours. Cells were subsequently washed and plated on YPD to determine percent survival and on selective media to identify recombinants. For all panels, error bars represent the S.E. of at least three trials.
A

KP1019 \(\mu\)g/ml

1.0

0.8

0.6

0.4

0.2

0.0

relative growth

B

Rad52-GFP

DAPI

DIC

0.1

1

10

100

Fig. 4. Recombination repair contributes to KP1019 tolerance. (A) wild-type (circles) and rad52D (triangles) yeast were grown for 19–24 hours in the indicated concentration of KP1019 as described under Materials and Methods. Error bars represent S.D. of three independent trials. (B) representative microscopy of Rad52-GFP yeast treated with 200 \(\mu\)g/ml KP1019 for 1 hour at 23°C. Nuclear and mitochondrial DNA were visualized with DAPI staining. Arrows point to Rad52-GFP foci. Calibration bar = 5 \(\mu\)m. (C) Rad52-GFP yeast were incubated with the indicated concentrations of KP1019 for 1 hour at 23°C prior to visualization of foci by fluorescence microscopy. Error bars represent the S.D. of three trials; 100 cells were counted per trial. (D) Rad52-GFP yeast were incubated with 0\(\mu\)g/ml (black bars) or 33\(\mu\)g/ml (gray bars) KP1019 for 1 hour at 23°C. For each trial, 100 cells were analyzed, with an average of 33 cells per stage of the cell cycle. Error bars represent the S.D. of three independent trials. Asterisks indicate statistically significant differences in Rad52-GFP focus formation relative to the 0 \(\mu\)g/ml control. *\(P < 0.05\), **\(P < 0.01\), ***\(P < 0.001\).

KP1019 May Be a Weak Topoisomerase II Poison in S. cerevisiae. Previous research suggests that KP1019 may be a topoisomerase II poison (Vashisht Gopal and Kondapi, 2001). Since such poisons block the reannealing step required for topoisomerase II mediated relaxation of DNA supercoils, resulting in double-strand breaks (Watt and Hickson, 1994), we wanted to determine whether the KP1019-induced recombination (Fig. 3C) might be the result of double-strand breaks caused by poisoning topoisomerase II. Because topoisomerase poisons turn this target enzyme into a DNA damaging agent, cells expressing high levels of topoisomerase are hypersensitive to poisons (Nitiss et al., 1992). To determine whether increased expression of the yeast topoisomerase II encoding gene TOP2 increased sensitivity to KP1019, we transformed wild-type yeast with the high-copy TOP2 plasmid (Nitiss et al., 1992). As seen in Fig. 5A, increased expression of TOP2 dramatically increases sensitivity to the well established topoisomerase II poison amsscarine. In contrast, yeast overexpressing TOP2 are only slightly sensitive to KP1019 (Fig. 5B), indicating that this ruthenium complex is, at best, a weak Top2 poison in living cells.

Disruption of Nucleotide Excision Repair and Translesion Synthesis Increases KP1019 Sensitivity. Having implicated RAD52 and therefore recombination as an important factor in modulating sensitivity to KP1019, we assessed the role of other DNA repair pathways in cell tolerance of KP1019. Specifically, by examining rev3Δ and rad1Δ strains, we determined that translesion synthesis (TLS) and nucleotide excision repair (NER), respectively, contribute to cellular resistance to KP1019 (Fig. 6A). In contrast, we found that deletion of genes central to mismatch repair (MSH2) and base excision repair (NTG1, NTG2, APN1) had little impact on drug sensitivity. Because mismatch repair and base excision repair play roles in fixing replication errors and small scale damage to individual bases, our data are consistent with previous studies indicating that KP1019 causes interstrand cross-links and protein-DNA adducts (Frühauf and Zeller, 1991), which are handled by TLS, NER, and recombination. Because TLS has been shown to be responsible for the mutations induced by the cross-linking agents cisplatin and nitrogen mustard (Beljanski et al., 2004), we examined the ability of KP1019 to induce mutations in rev3Δ yeast. As seen in Fig. 6B, KP1019 had little effect on the mutation frequency of the TLS-defective (rev3Δ) strain, suggesting that the error-
prone TLS pathway is responsible for the KP1019-induced mutations in wild-type yeast. The increased frequency of KP1019-induced mutation in the rad1Δ strain, suggests that in the absence of NER, KP1019 adducts are processed by more error-prone pathways such as TLS. The recombination-defective rad52Δ strain yielded highly variable results that were not significantly different from the wild-type or the TLS-compromised strain (data not shown).

**KP1019 Sensitivities of Strains Lacking Multiple Repair Pathways.** To gain additional insight into the mechanisms by which DNA repair and damage tolerance pathways may collaborate in processing KP1019 adducts, we examined the sensitivity of strains defective in two different pathways. As seen in Fig. 7A, deletion of NER or TLS alone increases KP1019 sensitivity, whereas yeast defective in both pathways are even more sensitive to the drug. Likewise, in Fig. 7B, the effects of disrupting recombination and TLS appear to be additive, as the double mutant is more sensitive than either single mutant. Interestingly, yeast lacking both recombination and NER are not any more sensitive to KP1019 than strains defective solely for recombination (Fig. 7C).

These findings suggest that Rad1 and Rad52 may both function within a single, larger pathway required for tolerance of KP1019-induced DNA damage, whereas TLS may function in a separate pathway.

**KP1019 Retains Potency against Pleiotropic Drug-Resistant S. cerevisiae.** Since KP1019 maintains potency against multidrug resistant (MDR) cancer cell lines (Heffeter et al., 2005), we wanted to establish whether KP1019 functions similarly when applied to S. cerevisiae exhibiting pleiotropic drug resistance (PDR), the yeast analog of MDR. In yeast, PDR is controlled primarily by two transcription activators, Pdr1 and Pdr3 (reviewed in Balzi and Goffeau, 1995; Moye-Rowley, 2003). These zinc-finger proteins bind to PDR response elements in the promoters of many genes, including several that encode ATP-binding cassette (ABC) transporters that efflux a diverse array of drugs from the cell. As seen in Fig. 8, we were able to confirm that the gain-of-function alleles PDR1-11 (Kean et al., 1997) and PDR1-3
increase resistance to the translation inhibitor cycloheximide. Consistent with observations in MDR cancer cell lines, both \textit{PDR1} mutants were hypersensitive to KP1019 (Fig. 8). These findings suggest that yeast could serve as a useful model organism for studying the mechanism by which MDR cancer cell lines remain sensitive to KP1019.

\textbf{Discussion}

Here we show that the budding yeast \textit{S. cerevisiae} can serve as an effective model organism for elucidating the molecular mechanism(s) by which the anticancer ruthenium complex KP1019 inhibits cell growth and induces cell death. Consistent with work in mammalian cells (Hartinger et al., 2008), Figs. 1 and 2 show that KP1019 exerts both cytotoxic and cytostatic effects on yeast. Although the concentration of KP1019 that kills approximately 50\% of wild-type yeast (163\(\mu\)g/ml or 273\(\mu\)M) is somewhat higher than the IC\textsubscript{50} values (56–179\(\mu\)M) reported for cancer cells in vitro (Heffeter et al., 2005; Heffeter et al., 2010), this result is not surprising given that yeast often display higher levels of resistance to antineoplastic agents (Stepanov et al., 2008).

KP1019\'s effects on cell growth and survival are likely to stem, at least in part, from the drug\'s genotoxicity. The ability of KP1019 to mutate the CAN1 locus and to induce the RNR3-lacZ reporter construct (Fig. 3), clearly demonstrate that KP1019 damages DNA (Whelan et al., 1979; Jia et al., 2002; respectively). This finding suggests that the binding of KP1019 to DNA is central to the drug\'s toxicity, whereas its reported binding to cytoplasmic components (Heffeter et al., 2010) may be secondary. Given that KP1019 treatment led to a dose-dependent increase in the proportion of large budded cells with nuclei that span and/or are near the bud neck and that cells treated with KP1019 continued to grow during this cell cycle delay (Fig. 2), we speculate that the KP1019-induced cell cycle delay occurs in response to DNA damage. In fact, the KP1019-induced G2/M arrest is likely to be caused by a mechanism similar to that which causes the G2/M arrest observed for cisplatin and nitrogen mustard–treated yeast (Beljanski et al., 2004). Future studies might address the mechanism of this cell cycle delay by looking for activation of checkpoint kinases, including Rad53, the yeast ortholog of human Chk2 (Nyberg et al., 2002).

The ability of KP1019 to induce recombination (Fig. 3C) and Rad52-GFP focus formation (Fig. 4) in yeast suggests that the drug is generating double-strand breaks that are tolerated via recombination (Symington, 2002). This finding is also

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig7.png}
\caption{KP1019 sensitivity of strains lacking multiple DNA repair pathways. Genetic interactions between (A) NER and TLS, (B) recombination and TLS and (C) recombination and NER were assessed by growing the relevant yeast strains for 18–24 hours in the indicated concentrations of KP1019 as described for Fig. 4A. Error bars represent S.D. of three independent trials.}
\end{figure}

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig8.png}
\caption{KP1019 maintains potency against PDR yeast. As described for Fig. 6, relative resistance was calculated by dividing each mutant\'s IC\textsubscript{50} for a given drug by the IC\textsubscript{50} for the corresponding wild-type control. Error bars represent the S.D. of three independent trials. Asterisks indicate statistically significant differences in drug resistance relative to the wild-type control. **\(P < 0.01\), ***\(P < 0.001\).}
\end{figure}
consistent with that of Kapitza et al. (2005), who showed that KP1019 treatment results in DNA breaks in HT29 colon carcinoma cells. Whereas KP1019-induced hydrogen peroxide production and the resulting oxidative stress have been proposed to be central to KP1019s ability to damage DNA, our data suggest otherwise. Oxidative stress typically leads to the formation of 8-oxoguanine, a toxic base that is processed primarily by base excision repair (Slupphaug et al., 2003), disruption of which has little effect on KP1019 sensitivity (Fig. 6A). Future studies might address whether the KP1019-induced oxidative stress observed in colon carcinoma cells (Kapitza et al., 2005) is limited to specific cell lines.

Although we cannot conclusively rule out compensatory activation of alternate DNA repair mechanisms, the relative lack of KP1019 sensitivity observed in strains with disabled mismatch repair and base excision repair (Fig. 6) argues against the drug inducing small-scale changes to DNA structure. Instead, our data are consistent with those from in vitro studies showing that KP1019 leads to interstrand cross-links and protein-DNA cross-links (Fruhaufl and Zeller, 1991). Previous studies have shown that KP1019 poisons topoisomerase II in biochemical assays (Vashisht Gopal and Kondapi, 2001). Unlike topoisomerase inhibitors that reduce the enzymes ability to cleave DNA, topoisomerase poisons prevent DNA religation, which results in lingering double-strand breaks and leaves the enzyme attached to DNA as a bulky adduct (Watt and Hickson, 1994). Thus, yeast with increased expression of TOP2 have increased sensitivity to topoisomerase II poisons (Nitiss et al., 1992). As shown in Fig. 5B, increased TOP2 expression has little effect on sensitivity to KP1019. We suspect that KP1019s ability to poison topoisomerase II strongly in vitro but only weakly in vivo may be due to the drugs inherent chemical reactivity, which allows it to bind to a wide range of macromolecules, including multiple proteins as well as model nucleotides (Hartner et al., 2008).

In many respects, KP1019 appears to act similarly to cisplatin. For example, Beljanski et al. (2004) reported that TLS, NER, and recombination all play important roles in cell tolerance of cisplatin. Moreover, yeast defective for both recombination and TLS are extremely sensitive to cisplatin. These findings are similar to our results (Fig. 6A and 7B). Given this similarity to cisplatin, it is likely that mechanisms of cisplatin resistance may also be relevant for KP1019. For example, increased expression of the mammalian NER pathway component ERCC1 is associated with a poor clinical prognosis as well as platinum resistance in vitro (Martin et al., 2008). Thus, elevated expression of ERCC1 and other elements of the NER pathway should be examined as a possible modulator of KP1019 resistance. Likewise, as combination therapy is commonly used when using platinum-based drugs to treat ovarian and non–small cell lung cancers, complementary chemotherapeutics should also be explored for KP1019. For example, the nucleotide analog gemicitabine has been found to be synergistic with cisplatin in some cell lines (Bergman et al., 1996). Similar effects may be seen upon administering both gemicitabine and KP1019. Such a model seems plausible given that gemicitabine inhibits ribonucleotide reductase, an enzyme complex induced in response to DNA damage both in yeast (Jia et al., 2002; Fig. 3A) and mammalian cell lines (Guittet et al., 2001).

One intriguing feature of KP1019 is that it maintains potency against many drug-resistant cell lines (Heffeter et al., 2005). To determine whether this feature is conserved between yeast and mammalian cells, we examined the ability of KP1019 to inhibit growth of yeast carrying mutations in the PDR network. The PDR network is comprised of two zinc finger transcription activators, Pdr1 and Pdr3, which regulate expression of a wide range of genes, including the ABC transporter–encoding genes, PDR5 and YOR1 (Sipos and Kuchler, 2006). The drug pumps Pdr5 and Yor1 efflux a wide range of xenobiotics out of the cell and are orthologous to the human ABCG2 (BCRP) and ABCCl (MRP1) ABC transporters, respectively (Paumi et al., 2009). Yeast strains with hypermorphic mutations in PDR1 and PDR3 overexpress a variety of ABC transporters and are resistant to a wide range of drugs, including cycloheximide (Rogers et al., 1999). Future studies might address whether the elevated KP1019 sensitivity observed in PDR yeast correlates with increased intracellular accumulation of the drug. Along the same lines, yeast might be used as a model organism for following up on KP1019s proposed role as a modulator of ABC transporter function and thus daunomycin resistance and rhodamine 123 accumulation (Heffeter et al., 2005).

Although targeted candidate gene approaches can be used to follow up on observations made to date, the greatest power of this model system lies in the wide array of unbiased genomic approaches that can be applied to elucidate KP1019s mechanism of action (Hughes, 2002). For example, although the transferrin pathway has been implicated as a mechanism of KP1019 internalization (Pongratz et al., 2004; Heffeter et al., 2005), our data demonstrate that the drug is capable of entering cells that lack the machinery for transferrin receptor–mediated endocytosis (Fig. 1). Thus, a genetic screen in yeast might shed light on the mechanism of KP1019 uptake as it did for cisplatin (Iahida et al., 2002). Yeast-based pharmacogenomic approaches will provide an affordable and efficient approach to better characterizing this clinically promising anticancer drug and perhaps also KP1019s sodium salt, KP1339.

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