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

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Abbreviations:
KP1019, trans-[tetrachlorobis(1H-indazole)ruthenate(III)], BER, base excision repair; NER, nucleotide excision repair; MMR, mismatch repair; TLS, translesion synthesis; PDR, pleiotropic drug resistance; YPD, medium composed of 1% yeast extract, 2% Bacto-peptone, 2% dextrose, 2% agar; SDC, medium composed of 0.67% yeast nitrogen base without amino acids, 2% dextrose, 0.2% drop-out mix, 2% agar.
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 <i>Saccharomyces cerevisiae</i> 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 crosslinks 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 <i>S. cerevisiae</i>. Whereas gain-of-function mutations in the transcription activator-encoding gene <i>PDR1</i> 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 <i>PDR1-11</i> or <i>PDR1-3</i>. Combined, these data suggest that <i>S. cerevisiae</i> 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 nephro-, neuro-, gastroentero- 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 (Allardyce and Dyson, 2001; Clarke et al., 1999; Zhang and Lippard, 2003). One such promising chemotherapeutic agent is the anticancer ruthenium complex \textit{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 \textit{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 lines resistant to doxorubicin and adriamycin remain sensitive to the effects of KP1019 (Heffeter et al., 2005).

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’s toxicity was largely ablated by addition of anti-oxidants, 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 crosslinks, indicating that the drug may damage DNA directly. Moreover, biochemical DNA relaxation assays have demonstrated that KP1019 acts as a topoisomerase II poison \textit{in vitro}, ultimately forming a drug-containing cleavage complex (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 predominantly in the cytosolic fraction of treated cells and that the ruthenium shifts from the high to low molecular weight fractions over time (Heffeter et al., 2010). These findings suggest that KP1019 has diverse targets, a model consistent with recent reports on the mechanism of action of other metal-based chemotherapeutic agents such as cisplatin. Specifically, cisplatin has been proposed to target not only DNA, but also the endoplasmic reticulum and the plasma membrane (Rebillard et al., 2008).

Due to the lack of clarity regarding KP1019’s mechanism of action, we wanted to determine whether the budding yeast \textit{Saccharomyces cerevisiae} might serve as an appropriate model organism for identifying genes and pathways that modulate sensitivity to the drug. In the past, numerous yeast studies have verified that DNA is a major target of cisplatin (Beljanski et al., 2004; Liao et al., 2007). A genetic screen in yeast was also used to successfully identify the evolutionarily conserved copper transporter Ctr1 as a mediator of cisplatin uptake (Ishida et al., 2002). Furthermore, a yeast genomic approach showed that loss of lysosomal/vacuolar proton transport increases cisplatin sensitivity (Liao et al., 2007), a result consistent with observations that some cisplatin resistant cancer cell lines overexpress V-type ATPases (Murakami et al., 2001). Likewise, \textit{S. cerevisiae} has been used to gain insight into the mechanisms of diverse antineoplastic agents including the topoisomerase I inhibitor camptothecin and the angiogenesis inhibitor fumagillin (Cardenas et al., 1999).

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 media (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 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 *rad1*Δ (DSC0036) yeast using lithium acetate as described above. Putative deletion strains were isolated in YPD + 200μg/ml G418. Genotypes were confirmed by PCR.

**Drug Synthesis.** KP1019 was synthesized using a protocol adapted from Lipponer et al. (1996). Briefly, 1g RuCl₃•3H₂O was added to 20 ml of 12M HCl and 20 ml of ethanol. The solution was then refluxed for one hour then cooled to 40°C. The ethanol was then removed from the solution through the use of a rotary evaporator. 12M HCl was added to a final volume of 40 ml. 1.87g of indazole was heated to 70°C in 30 ml 12M 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 then stirred in approximately 75 mL of H₂O at room temperature for 2 hours. The solid was filtered and subsequently washed with cold ethanol and lastly cold diethyl ether. The drug was dried under vacuum for 18-24 hours. KP1019 purity was verified by UV-vis 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-vis 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 OD$_{600}$ of 0.5-0.6. 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 2 hours at 30°C. Following drug treatment, cells were washed three times with water, 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. *S. cerevisiae* strain SJR0751 was used for survival and mutation assay, and *can1* mutants were identified by plating on SDC-arg containing 60μg/ml canavanine. *S. cerevisiae* strain SJR0897 was used for analysis of drug-induced recombination; *lys2* recombinants were identified by plating on SDC-lys.

**KP1019 Uptake.** Wild-type yeast (BY 4742) were grown in SDC until mid-log phase (OD$_{600}$ approximately 0.8). This culture was then split into four 20ml aliquots that were each diluted two-fold with fresh media either lacking or containing KP1019 (20μg/ml final concentration). One of the resulting 40ml KP1019-containing samples was pelleted immediately, washed, and ashed as described below. The two remaining KP1019-containing samples and the 0μg/ml KP1019 control were incubated for two 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 conducted on three independent cultures of wild-type yeast. To prepare the cell pellets for ashing, each yeast sample was resuspended in a minimal volume of distilled water and spread into porcelain weigh boats. Samples were then transferred to a tube furnace wherein the temperature was gradually raised from 200 to 500°C over the span of approximately 5 hours. Ashing continued at 500°C for 5 hours. Cooled ashes were dissolved in 1.8 mL nitric acid and 3.2 mL deionized water. Dissolved
ashes were analyzed by atomic absorption spectroscopy using a Perkin-Elmer AAnalyst 800 with a graphite furnace and a ruthenium lamp. Each biological sample was analyzed in triplicate and compared to a standard curve (0-99 ppb), samples for which were also read three times each.

**Drug Sensitivity.** Drug-containing SDC was subjected to two-fold serial dilution across the wells of a microtitre plate. Overnight cultures of yeast were diluted to OD$_{600}$ = 0.1 and subsequently diluted 20-fold more in SDC. An equal volume of diluted cell suspension was added to each well of the microtitre plate. After an 18-24 hour incubation at 30°C, the growth of each strain at each concentration of drug was recorded as absorbance at 630nm using a BioTek microtitre plate reader.

**Cell Cycle Analysis.** *S. cerevisiae* strain JBY206-1C was grown to early log phase (OD$_{600}$ 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% output on Sonicator Dismembrator Model 100 (Fisher Scientific) to disrupt cellular clumps, and placed directly on a microscope slide for image capture. Images were captured using a 20X objective with the LSM710 Confocal Imaging System (Zeiss, Inc) with ZEN software (Zeiss, Inc). DIC and fluorescent 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 300 and 500 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. To measure changes in cell size of the budded cell population specifically, the threshold for area measurement was set between 30-80μm$^2$ for all images in order 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.
were made from three independent time courses for each treatment dosage. The same images were analyzed for both cell size, cellular and nuclear morphology.

**Beta-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 (OD$_{600}$ 0.5-1.0) in selective media then treated with KP1019 (0, 20, 40, 60, 80, or 100μg/ml) dissolved in the same type of medium. Samples were incubated for three 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 beta-galactosidase activity by the activity measured for the 0μg/ml KP1019 control.

**Analysis of Rad52-GFP localization.** Overnight cultures of Rad52-GFP yeast were sub-cultured to mid log phase (OD$_{600}$ 0.5-1.0) in SDC at 23°C. KP1019 was dissolved in SDC at a concentration of 1mg/ml 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 1μg/ml. GFP and DAPI were visualized using the 100X objective of a Zeiss Axioskop 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 dose dependent increase in cell death when the budding yeast Saccharomyces cerevisiae was treated with KP1019 (Fig. 1A). Given that KP1019 has been reported to be internalized by the transferrin pathway (Pongratz et al., 2004; Kratz et al., 1994) 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 Figure 1B, a dramatic increase in cell-associated ruthenium was observed when yeast cells were incubated with 20μg/ml KP1019 for two 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 Figure 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 one hour, all of the KP1019 treated samples were significantly different from the no drug control (p<0.05). By three 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 to 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 (Kapitza et al., 2005; Frühauf and Zeller, 1991). 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 Figure 3A, KP1019 treatment induces *RNR3-lacZ* expression in a dose dependent fashion. This increase in expression peaks at 40μg/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). Figure 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 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 Figure 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 Figure 4A, deletion of *RAD52* dramatically increases KP1019 sensitivity. Specifically, the IC$_{50}$ for the wild-type strain is 5.0μg/ml whereas the IC$_{50}$ for the *rad52Δ* strain is 0.53μg/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). Figure 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 the Rad52-GFP focus formation experiments (data not shown).

**KP1019 may be a weak topoisomerase II poison in S. cerevisiae.** Previous research suggests that KP1019 may be a topoisomerase II poison (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 Figure 5A, increased expression of TOP2 dramatically increases sensitivity to the well established topoisomerase II poison amsacrine. 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 NER and TLS 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 (\textit{MSH2}) and base excision repair (\textit{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 crosslinks 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 crosslinking agents cisplatin and nitrogen mustard (Beljanski et al., 2004), we examined the ability of KP1019 to induce mutations in \textit{rev3\textDelta} yeast. As seen in Figure 6B, KP1019 had little effect on the mutation frequency of the TLS-defective (\textit{rev3\textDelta}) 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 \textit{rad1\textDelta} strain, suggests that in the absence of NER, KP1019 adducts are processed by more error-prone pathways such as TLS. The recombination-defective \textit{rad52\textDelta} strain yielded highly variable results that were not significantly different from the wild-type nor 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 Figure 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 Figure 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 PDR S. cerevisiae.** Since KP1019 maintains potency against multi-drug 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 (the yeast analog of MDR). In yeast, pleiotropic drug resistance (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 (PDREs) 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 Figure 8, we were able to confirm that the gain-of-function alleles *PDR1-11* (Kean et al., 1997) and *PDR1-3* (Rogers et al., 2001) increase resistance to the translation inhibitor cycloheximide. Consistent with observations in MDR cancer cell lines, both *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.
Discussion

Here we show that the budding yeast *Saccharomyces 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), Figures 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 μg/ml or 273 μM) is somewhat higher than the IC₅₀ values (56-179 μM) 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 induce the *RNR3-lacZ* reporter construct and to mutate the *CAN1* locus (Fig. 3), clearly demonstrate that KP1019 damages DNA (Jia et al., 2002; Whelan et al., 1979; 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 consistent with 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 KP1019’s ability to damage DNA, our data suggest otherwise, because 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.

While 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 in vitro studies showing that KP1019 leads to interstrand crosslinks (ICLs) and protein-DNA crosslinks (Frühauf and Zeller, 1991). Previous studies have shown that KP1019 poisons topoisomerase II in biochemical assays (Gopal and Kondapi, 2001). Unlike topoisomerase inhibitors which reduce the enzyme’s 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 Figure 5B, increased TOP2 expression has little effect on sensitivity to KP1019. We suspect that KP1019’s ability to poison topoisomerase II strongly in vitro but only weakly in vivo may be due to the drug’s inherent chemical reactivity which allows it to bind to a wide range of macromolecules, including multiple proteins as well as model nucleotides (Hartinger 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 comparable 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 *ERCCI* is associated with a poor clinical prognosis as well as platinum resistance *in vitro* (Martin et al., 2008). Thus elevated expression of *ERCCI* 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 gemcitabine has been found to be synergistic with cisplatin in some cell lines (Bergman et al., 1996). Similar effects may be seen upon administering both gemcitabine and KP1019. Such a model seems plausible given that gemcitabine 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 pleiotropic drug resistance (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 ATP-binding cassette (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 ABCC1 (MRP1) ABC transporters, respectively (Paumi et al., 2009). Yeast strains with hypermorph 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., 2001; Moye-Rowley, 2003; Fig. 8). Here we have shown that the hyperactive *PDR1-3* and *PDR1-11* alleles actually increase sensitivity to KP1019 (Fig. 8). The fact that the KP1019 sensitivity was not allele-specific suggests that the KP1019 sensitivity may be a general feature of pdr yeast. Although these findings parallel what has been observed in mammalian cell lines (Heffeter et al., 2005), they are surprising given the role the pdr network plays in effluxing and detoxifying heavy metals (Bauer 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 KP1019’s 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 KP1019’s 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 (Ishida 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 KP1019’s sodium salt KP1339.
Acknowledgments

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Authorship Contributions

Participated in research design: Stevens, Strehle, R.L. Miller, Gammons, Hoffman, McCarty, M.E. Miller, and Hanson

Conducted experiments: Stevens, Strehle, R.L. Miller, Gammons, Hoffman, McCarty, M.E. Miller, and Hanson

Contributed new reagents or analytic tools: Stultz

Performed data analysis: Stevens, Strehle, R.L. Miller, Gammons, Hoffman, McCarty, M.E. Miller, and Hanson

Wrote or contributed to writing of the manuscript: Hanson and M.E. Miller
References


Footnotes

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Meeting Abstracts:


Exposure to ruthenium-based chemotherapeutic KP1019 delays cell cycle progression in S. cerevisiae. Southeastern Regional Yeast Meeting, Mississippi State University, March 11-13, 2011.


The anti-cancer ruthenium complex KP1019 induces Rad52-GFP focus formation. Southeastern Regional Yeast Meeting, Vanderbilt University, March 27-29, 2009.


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Fig. 1. KP1019 kills yeast. A, as described in the Materials and Methods, Wild-type yeast (SJR0751) were treated with the indicated concentrations of KP1019 for two 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 (BY 4742) 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 in the Materials and Methods. A, representative microscopy showing accumulation of cells showing nuclei spanning and/or near bud neck. Size bar = 10 microns. 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 (grey) and 60 μg/mL drug (black). Error bars represent 2X standard error of three experimental trials. Asterisk indicates statistically significant difference in cell size relative to the 0 μg/ml control, *p < 0.05.

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 β-galactosidase activity. B, the frequency of KP1019-induced mutation was measured by treating wild type strain SJR0751 with KP1019 for two 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 two 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 standard error of at least three trials.

**Fig. 4.** Recombination repair contributes to KP1019 tolerance. A, wild-type (circles) and rad52Δ (triangles) yeast were grown for 18-24 hours in the indicated concentration of KP1019 as described in Materials and Methods. Error bars represent standard deviation of three independent trials. B, representative microscopy of Rad52-GFP yeast treated with 200 μg/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 = 5μm  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 standard deviation of three trials; 100 cells were counted per trial. D, Rad52-GFP yeast were incubated with 0μg/ml (black bars) or 33μg/ml (grey 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 standard deviation of three independent trials. Asterisks indicate statistically significant differences in Rad52-GFP focus formation relative to the 0 μg/ml control, *p < 0.05, **p < 0.01, ***p<0.001.

**Fig. 5.** KP1019 may be a weak topoisomerase II poison. Wild-type yeast (BY4742) transformed with a high copy TOP2 plasmid (squares) or an empty vector (circles) were grown for 18-24 hours in SDC-ura containing the indicated concentration of KP1019. Growth was documented as OD630 using a microtitre plate reader. In three independent trials, TOP2 overexpression resulted in a greater increase in sensitivity to amsacrine than KP1019. However, the absolute values of these differences varied from trial to trial, presumably due to variation in plasmid copy number. Thus, only one representative trial is shown for each drug.
**Fig. 6.** NER and TLS contribute to KP1019 tolerance. A, dose response curves were constructed as described for Figure 4A. The resulting IC\textsubscript{50} values were used to calculate relative resistance by dividing each mutant’s IC\textsubscript{50} by the IC\textsubscript{50} of the corresponding wild-type strain. Error bars represent the standard deviation of at least three independent trials. Asterisks indicate statistically significant differences in KP1019 sensitivity relative to the wild-type control, ***p<0.001. B, the frequency of KP1019-induced mutation of the \textit{CAN1} gene was measured as described for Figure 3B. Error bars represent the standard error of three independent trials.

**Fig. 7.** KP1019 sensitivity of strains lacking multiple DNA repair pathways. Genetic interactions between NER and TLS (A), recombination and NER (B), and recombination and TLS (C) were assessed by growing the relevant yeast strains for 18-24 hours in the indicated concentrations of KP1019 as described for Figure 4A. Error bars represent standard deviation of three independent trials.

**Fig. 8.** KP1019 maintains potency against PDR yeast. As described for Figure 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 standard deviation of three independent trials. Asterisks indicate statistically significant differences in drug resistance relative to the wild-type control, **p < 0.01, ***p<0.001.
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<td>SJR0751</td>
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<td>JB206-1C</td>
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<td>msh2::kanMX</td>
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<td>Rad52-GFP</td>
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Figure 1

A

B

percent survival

[KP1019] (µg/ml)

[ppb Ru/OD600]

0 µg/ml 0 hr 2 hrs

20 µg/ml

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Figure 2

A

merge  histone H2B-mCherry  DIC

0 µg/ml

60 µg/ml

B

% large budded cells with nuclei at bud neck

0 1 2 3

0 10 20 30 40 50 60 70

time (hours)

C

average cell area

0 10 20 30 40 50 60 70

0 µg/mL  60 µg/mL

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Figure 4

A

[Graph showing relative growth vs. KP1019 (µg/ml)]

B

[Images of Rad52-GFP, DAPI, and DIC of cells with arrows indicating foci]

C

[Bar graph showing % cells with foci at different KP1019 concentrations]

D

[Bar graph showing % cells with foci at different bud sizes for control and KP1019]
Figure 5

A

B

amsacrine (mg/ml)

relative growth

0.0
0.2
0.4
0.6
0.8
1.0

0.01
0.1
1

0.001

KP1019 (µg/ml)

relative growth

0.0
0.2
0.4
0.6
0.8
1.0

0.1
1
10
100
Figure 6

A

B

relative resistance to KP1019

wild-type  rad52Δ  rad1Δ  rev2Δ  msh2Δ  ntg1Δ  ntg2Δ  apr1Δ

Can^r frequency over background (x10^-6)

KP1019 (µg/ml)

0 50 100 150 200

Can^r frequency over background (x10^-6)

wild-type  rad1Δ  rev2Δ

0 5 10 15 20 25 30 35 40

***

***

***
Figure 7

(A) Relative growth of wild-type, rad1Δ, rev3Δ, and rad1Δ rev3Δ strains in response to different concentrations of KP1019 (µg/ml).

(B) Relative growth of wild-type, rad52Δ, rev3Δ, and rad52Δ rev3Δ strains in response to different concentrations of KP1019 (µg/ml).

(C) Relative growth of wild-type, rad1Δ, rad52Δ, and rad1Δ rad52Δ strains in response to different concentrations of KP1019 (µg/ml).
Figure 8