

Inactivation of the *Saccharomyces cerevisiae* SKY1 Gene Induces a Specific Modification of the Yeast Anticancer Drug Sensitivity Profile Accompanied by a Mutator Phenotype

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ABSTRACT

The therapeutic potential of the highly active anticancer agent cisplatin is severely limited by the occurrence of cellular resistance. A better understanding of the molecular pathways involved in cisplatin-induced cell death could potentially indicate ways to overcome cellular unresponsiveness to the drug and thus lead to better treatment results. We used the budding yeast *Saccharomyces cerevisiae* as a model organism to identify and characterize novel genes involved in cisplatin-induced cell kill, and found that SKY1 (SR-protein-specific kinase from budding yeast) is a cisplatin sensitivity gene whose disruption conferred cisplatin resistance. In cross-resistance studies, we observed resistance of yeast *sky1Δ* cells (i.e., cells from which the SKY1 gene had been disrupted) to cisplatin, carboplatin

(but not oxaliplatin), doxorubicin and daunorubicin, and hypersensitivity to cadmium chloride and 5-fluorouracil. Furthermore, these cells did not display reduced platinum accumulation, DNA platination or doxorubicin accumulation, indicating that the resistance is unrelated to decreased drug import or increased drug export. Based on the modification of the anticancer drug sensitivity profile and our finding that *sky1Δ* cells display a mutator phenotype, we propose that Sky1p might play a significant role in specific repair and/or tolerance pathways. Disruption of the *S. cerevisiae* SKY1 gene would thus result in deregulation of such mechanisms and, consequently, lead to altered drug sensitivity.

Intrinsic or acquired resistance to cisplatin is frequently encountered and severely limits the therapeutic potential of this drug, which is highly active against cancers of the lung, ovary, bladder, head and neck, esophagus, cervix, and endometrium, and curative for most patients with testicular cancer (Kelland, 1994; Einhorn, 1997). It is generally believed that a better understanding of the pathways leading to cisplatin-induced cell death might shed light on putative mechanisms operative in the cisplatin resistance phenotype.

Ample evidence indicates that cisplatin exerts its cytotoxic action by the formation of platinum-DNA adducts. Although less abundant lesions such as the interstrand cross-links do play their parts, the predominant 1,2-intrastrand GpG and ApG cross-links (which represent approximately 90% of the total adducts) are generally thought to be the DNA lesions of most therapeutic significance (Pratt et al., 1994; Johnson et al., 1998). In vitro studies have revealed that the mechanisms by which cells may overcome the cytotoxic action of

cisplatin include decreased intracellular drug accumulation, inactivation by glutathione or metallothioneins (leading to reduced DNA platination), aberrations in repair, enhanced tolerance, and defects in pathways modulating cell death (Perez, 1998).

Paradoxically, repair of cisplatin-induced DNA damage has been implicated in both cisplatin resistance and sensitivity. The nucleotide excision repair (NER) system efficiently removes a broad spectrum of DNA lesions, including those produced by UV radiation and cisplatin. Enhanced NER playing a role in cisplatin resistance has been observed frequently (Crul et al., 1997), and reduced NER might account for the hypersensitivity of testicular germ cell tumors (Kelland, 1994). Whereas NER deficiency leads to cisplatin hypersensitivity, mismatch repair (MMR) defects make cells resistant to the drug (Crul et al., 1997; Vaisman et al., 1998). Two prominent models have been proposed to explain the latter phenomenon. First, binding of MMR proteins to cisplatin-DNA adducts might directly activate signal transduction pathways leading to cell cycle arrest and/or cell death. Alternatively, futile cycles of translesion synthesis past DNA le-

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ABBREVIATIONS: NER, nucleotide excision repair; MMR, mismatch repair; SKY1, SR-protein-specific kinase from budding yeast; PCR, polymerase chain reaction; AAS, atomic absorption spectrometry; HPLC, high-performance liquid chromatography; SR-protein, serine/arginine-rich protein; SRPK, SR-protein-specific kinase; NMU, *N*-nitroso-*N*-methylurea; MDR, multidrug resistance; PDR, pleiotropic drug resistance.

sions, followed by recognition and removal of the newly synthesized strand by an active hMutS α /hMutL α MMR protein complex, are thought to generate gaps or strand breaks that induce cell death. It is believed, therefore, that loss of MMR leads to increased replicative bypass of cisplatin adducts and reduced cell death and, thus, resistance (Crul et al., 1997; Vaisman et al., 1998).

Currently known in vitro mechanisms of cisplatin resistance do not fully account for the observed in vivo unresponsiveness of particular tumors to platinum-based chemotherapy (Perez, 1998). Hence, additional cisplatin resistance mechanisms, for which the genes involved still have to be identified, are believed to exist.

We are using the budding yeast *Saccharomyces cerevisiae* as a model system to study modulation of drug sensitivity. We have performed a genome-wide functional screen to identify and characterize novel cisplatin sensitivity genes, and found that disruption of the *S. cerevisiae* *SKY1* (SR-protein-specific kinase from budding yeast) gene conferred cellular resistance to cisplatin (Schenk et al., 2001). In the cross-resistance studies presented here, we observed resistance of yeast *sky1 Δ* cells (i.e., cells from which the *SKY1* gene had been disrupted) to cisplatin, carboplatin (but not oxaliplatin), doxorubicin, daunorubicin, and hypersensitivity to cadmium chloride and 5-fluorouracil. Based on the cytotoxicity data and our finding that *sky1 Δ* cells show a mutator phenotype, we propose that Sky1p might play a significant role in MMR, base excision repair, and/or Rev3p-dependent pathways. Disruption of *SKY1* would thus result in deregulation of repair and/or tolerance mechanisms and, consequently, lead to altered drug sensitivity.

Materials and Methods

Chemicals. Yeast Nitrogen Base and Yeast extract/Peptone/Dextrose Broth were purchased from DIFCO Laboratories (Detroit, MI). Cisplatin [Platosin, *cis*-diamminedichloroplatinum(II)] and doxorubicin hydrochloride (Doxorubin) were obtained from Pharmachemie (Haarlem, The Netherlands), carboplatin [Paraplatin, *cis*-diammine(1,1-cyclobutanedicarboxylato)platinum(II)] and etoposide (Vepesid) were from Bristol-Myers Squibb (Woerden, The Netherlands), and oxaliplatin [Eloxatin, [*trans*-(L)-1,2-diaminocyclohexane]oxalatoplatinum(II)] was from Sanofi-Synthelabo (Maassluis, The Netherlands). Procarbazine hydrochloride (Natulan) was obtained from Sigma-Tau Pharmaceuticals (Assen, The Netherlands). 6-Mercaptopurine was purchased from BUFA Pharmaceutical Products (Uitgeest, The Netherlands), and 5-fluorouracil (Fluorouracil-TEVA) from TEVA Pharma (Mijdrecht, The Netherlands). Other chemicals including cytotoxic agents and verapamil were obtained from Sigma-Aldrich (Zwijndrecht, The Netherlands).

Yeast Strains and Growth Conditions. *Saccharomyces cerevisiae* strains used in this study were NER- and recombinational repair-proficient W303-1B (*MAT α ho ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*), isogenic NER-deficient *rad4 Δ* mutant MGSC131 (*rad4 Δ ::hisG-URA3-hisG*) (Verhage et al., 1996) and isogenic recombinational repair-deficient *rad52 Δ* mutant MGSC194 (*rad52 Δ ::TRP1*). Uracil-deficient strain MGSC283 was derived from MGSC131 by selection on synthetic medium plates containing 5-fluoro-orotic acid (see below). Strain MGSC284 and MGSC186 were made by replacing the *ura3* allele of MGSC283 and W303-1B, respectively, with PCR-generated wild-type *URA3* sequence. Yeast strains were routinely grown on selective synthetic Yeast Nitrogen Base medium at 30°C as described previously (Burger et al., 2000). Although strain W303-1B is generally used as a wild-type yeast background, it has been reported that there is a spontaneous G-to-A

transition change in the W303 *RAD5* sequence, resulting in a weak *rad5-535* allele (Fan et al., 1996).

Isolation of Cisplatin-Resistant Yeast Strains. In a previous study, we transformed cisplatin-sensitive *S. cerevisiae* strain MGSC131 with a yeast genomic mini-Tn3::lacZ::LEU2 transposon insertion library (Burns et al., 1994). The cisplatin-resistant phenotype of transposon-derived colonies surviving a one-step drug selection at 4 μ g/ml cisplatin was confirmed in semiquantitative and quantitative cytotoxicity assays as described below. The sequences flanking the single transposon elements were then identified by inverse PCR and sequencing of the resulting PCR products employing transposon-specific primers, followed by comparison with public databases (Schenk et al., 2001). *S. cerevisiae* MGSC-*sky1 Δ* and W303-*sky1 Δ* cells containing a gene-specific *SKY1* disruption were generated by one-step gene replacement [i.e., transformation of strain MGSC283 and W303-1B, respectively, with a knockout construct obtained using primers SKY1-5A (5'-gTA AgA AAg CTg ggA Tgg ggC CAC TTC TCA TCT TTg ACA gCT TAT CAT C-3') and SKY1-3 (5'-Cgg AAC CAC TCC CgT ACA ACT CTC TAT CAg gTA CCC ACT CgT gCA CCC-3')]. Successful gene disruption was confirmed by PCR and Northern blotting as described previously (Schenk et al., 2001). *RAD52* was disrupted from W303-1B-derived *sky1 Δ* cells by transformation of *Bam*HI-linearized plasmid pSM21 as described previously (Siede et al., 1996). This yielded strain *rad52 Δ sky1 Δ* , for which successful disruption of the *RAD52* gene was confirmed by Southern blotting.

Construction of Expression Plasmids. A 2.3-kb *SKY1* PCR product was generated using primers SKYex-5 (5'-ATA gTg gAT CCT ggT ATA AAT AgA CAC CCC C-3') and SKYex-3 (5'-CTA ACC TCg AgA ggg CAA AAT AAA ggT ATA AAg g-3'), and cloned into the low-copy *S. cerevisiae* expression vector pYCTEF containing the constitutive translation elongation factor 1 α promoter (Schenk et al., 2001). Alternatively, the *SKY1* coding region was cloned into high-copy 2 μ -based yeast expression vector pEG202 (Gyuris et al., 1993) harboring the constitutive alcohol dehydrogenase promoter. The *SKY1* insert for pEG202 was made by PCR using primers SKY-ATG (5'-CAT ggA TCC ATA Tgg gTT CAT CAA TTA ACT ATC C-3') and SKYex-3.

Cytotoxicity Assays. Relative cisplatin or doxorubicin sensitivity was determined by a semiquantitative spot assay as described previously (Burger et al., 2000). Alternatively, suspensions of cells were streaked onto selective medium plates containing 0, 2, 3, 4, or 5 μ g/ml cisplatin and incubated at 30°C for 3 to 4 days. Sensitivity to cytotoxic chemicals was quantitatively analyzed by a clonogenic survival assay. Serial dilutions of mid-log phase yeast cells were plated onto selective medium containing various compound concentrations. Upon incubation at 30°C for 3 to 4 days, colonies were counted and percent survival was calculated based on the number of colonies arising in the absence of cytotoxic chemical. Sensitivity to ionizing radiation was tested by a similar assay, during which plating of the yeast cells was immediately followed by irradiation with increasing doses of γ rays (0, 50, 100, 200, 400 and 500 Gy) using opposing ¹³⁷Cs sources (Gamma Cell 40; Atomic Energy of Canada, Ottawa, Canada) at a rate of 1.06 to 1.08 Gy/min. Sensitivity to UV light was tested by a semiquantitative spot assay as described previously (Burger et al., 2000), using a germicidal lamp at 254 nm.

Determination of Intracellular Platinum Accumulation and DNA Platination. Cellular platinum content was measured by atomic absorption spectrometry (AAS) using a flameless spectrometer (4110 ZL; PerkinElmer, Foster City, CA) as described previously (Burger et al., 2000). Briefly, 2 \times 10⁷ exponentially growing yeast cells were incubated in 5 ml of selective liquid medium containing various concentrations of cisplatin (0, 5, 10, 20, and 40 μ g/ml) for 18 h and, after drug exposure, immediately washed three times. A total of 4 \times 10⁷ cells were then pelleted and lysed in 100 μ l of chloroform. After evaporation of residual chloroform, samples were dissolved in 0.2% nitric acid and subjected to AAS to determine total platinum content. DNA platination was measured by ³²P-postlabel-

ing. Briefly, 4×10^7 exponentially growing *S. cerevisiae* cells were incubated in 10 ml of selective liquid medium containing various concentrations of cisplatin (0, 5, and 40 $\mu\text{g/ml}$) for 18 h. Genomic DNA was then isolated and dissolved in 100 μl of H_2O , and GpG and ApG adduct levels were determined as described previously (Pluim et al., 1999).

Determination of Intracellular Doxorubicin Accumulation. Cellular doxorubicin content was measured by high-performance liquid chromatography (HPLC) as described previously (de Bruijn et al., 1999). Briefly, 8×10^6 exponentially growing yeast cells were incubated in 2 ml of selective liquid medium per Hemogard Vacutainer tube (Becton Dickinson, Franklin Lakes, NJ) containing various concentrations of doxorubicin (0, 30, 60, 120, and 240 $\mu\text{g/ml}$) for 18 h and, after drug exposure, immediately washed three times in ice-cold medium. A total of 2×10^7 cells were then pelleted, dissolved in drug-free human plasma, and subjected to pretreatment and HPLC as described previously (de Bruijn et al., 1999) to determine total doxorubicin content.

Determination of Mutation Rates. Spontaneous mutation rates were established by monitoring conversion of the *URA3*⁺ to the *ura3* mutant phenotype. For each strain tested, a total of 15 parallel cultures were inoculated at ~ 100 cells/ml and grown to stationary phase in liquid Yeast extract/Peptone/Dextrose Broth at 30°C. The viable titers and numbers of mutants were determined by plating different aliquots of the cultures on the appropriate media. Mutations in the *URA3* locus leading to abrogation of gene function were detected using synthetic medium plates containing 1 mg/ml 5-fluoroorotic acid as described previously (Boeke et al., 1984). Rates were calculated from the resulting median mutant frequencies as described previously (Drake, 1991).

Results

Isolation of Cisplatin-Resistant Yeast Strains. In order to identify yeast genes that, upon disruption, confer cisplatin resistance, we previously introduced a yeast genomic transposon insertion library (Burns et al., 1994) into *Saccharomyces cerevisiae* cells (Schenk et al., 2001). Transformation of yeast with this library corresponds to replacement of the original yeast genomic copy with the mutagenized version, by homologous recombination between yeast DNA transposon flanking sequences and endogenous genomic sequences. NER-deficient *rad4Δ* strain MGSC131 was used as recipient because it is hypersensitive to cisplatin and, thus, displays a steep dose-response curve to the drug (McA'Nulty and Lippard, 1996; Burger et al., 2000), enabling clear-cut selection of cisplatin-resistant colonies at a relatively low drug dose. When 3×10^5 library-derived transformants were screened and subsequently retested for cisplatin resistance, nine strains showing a 4-fold cisplatin resistance were selected for further characterization. It turned out that the YMR216C locus had been disrupted in at least five transposon-containing cisplatin-resistant yeast strains. From the different sizes of the inverse PCR products, we inferred that at least three of these strains had been derived from independent transformants (Schenk et al., 2001). In the remaining cisplatin-resistant yeast strains, other loci turned out to be disrupted, as will be described elsewhere (P. W. Schenk, M. Brok, A. W. M. Boersma, J. A. Brandsma, H. den Dulk, H. Burger, G. Stoter, J. Brouwer, K. Nooter, manuscript in preparation). The YMR216C locus corresponds to the *SKY1* (SR-protein-specific kinase from budding yeast) gene, which encodes a protein serine/threonine kinase with structural and functional homology to the human SR-protein-specific kinases (SR-PKs). The SR-protein-specific kinases and their substrates

(the serine/arginine-rich- or SR-proteins, including yeast Npl3p) are thought to be key regulators of RNA processing and, in mammalian cells, alternative splicing, through multiple mechanisms (Siebel et al., 1999; Yun and Fu, 2000).

Disruption of the *SKY1* Gene Renders Yeast Cells Cisplatin-Resistant. The cisplatin-resistant phenotype of the transposon containing yeast strains in principle could have arisen either from library-derived gene disruption or, despite the low dose of cisplatin used for selection, from cisplatin-induced mutations acquired during the screening procedure. Therefore, we independently disrupted the *SKY1* gene from the *S. cerevisiae* genome using PCR-generated one-step gene replacement constructs, and determined the cisplatin sensitivity profile of the resulting *sky1Δ* cells (Fig. 1A). Like transposon-derived *sky1Δ* transformants, MGSC-*sky1Δ* cells generated by one-step gene replacement were about 4-fold more cisplatin-resistant than MGSC131 *SKY1*⁺ cells (Schenk et al., 2001).

To further confirm that cisplatin resistance was linked to disruption of the *SKY1* gene, its coding region was cloned into *S. cerevisiae* expression vectors, and the resulting plasmids were transformed into *sky1Δ* or *SKY1*⁺ cells. When the

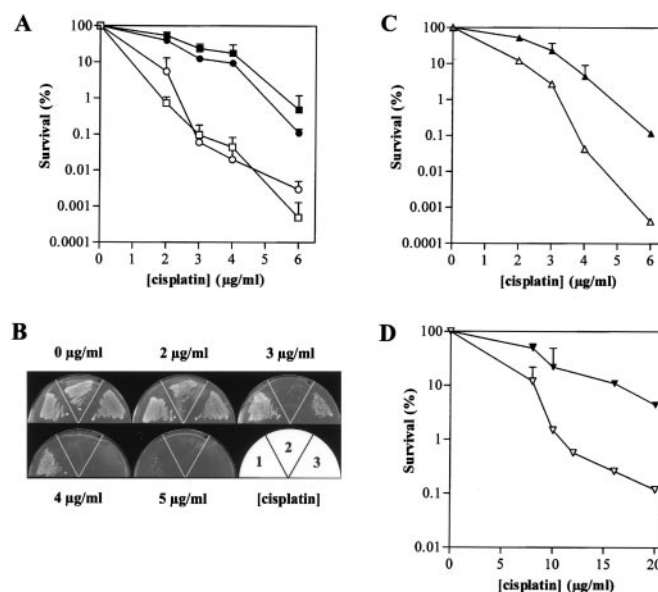


Fig. 1. Cisplatin sensitivity of untransformed yeast *sky1Δ* versus *SKY1*⁺ cells and *SKY1*-transformed cells. **A**, cisplatin sensitivity profiles of *S. cerevisiae* MGSC131 *SKY1*⁺ cells (□), isogenic MGSC-*sky1Δ* cells (●), MGSC-*sky1Δ* cells transformed with pYCTEF (■), and MGSC-*sky1Δ* cells transformed with pYCTEF-*SKY1* (○). pYCTEF is a low-copy yeast expression vector harboring the constitutive translation elongation factor 1 α promoter. Percentage survival (colony formation) at each concentration of cisplatin is expressed relative to untreated control cells (100%). Means of three independent experiments performed in triplicate are shown, with bars representing S.D. Bars are sometimes masked by the data point symbols. The data shown have been presented previously (Schenk et al., 2001). **B**, yeast transformants carrying high-copy plasmids were tested for their relative ability to grow on cisplatin by streaking onto selective medium plates containing the indicated drug concentrations. 1, *S. cerevisiae* MGSC-*sky1Δ* cells transformed with pEG202; 2, MGSC-*sky1Δ* cells transformed with pEG202-*SKY1*; 3, empty vector-transformed MGSC131 *SKY1*⁺ cells. pEG202 is a high-copy yeast expression vector harboring the constitutive alcohol dehydrogenase promoter. **C-D**, cisplatin sensitivity profiles of *S. cerevisiae* *rad52Δ SKY1*⁺ cells (**C**, Δ), isogenic *rad52Δ sky1Δ* cells (**C**, ▲), W303-1B-derived MGSC186 *SKY1*⁺ cells (**D**, ▽) and isogenic W303-*sky1Δ* cells (**D**, ▼). Percentage survival (colony formation) at each concentration of cisplatin is expressed relative to untreated control cells (100%). Means of typical experiments performed in triplicate are shown, with bars representing S.D..

low-copy plasmid pYCTEF-*SKY1* (with *SKY1* under the constitutive translation elongation factor 1 α promoter) was transformed into MGSC-*sky1* Δ cells, the cisplatin-resistant phenotype was fully complemented. The cells became as sensitive to cisplatin as the original MGSC131 *SKY1*⁺ strain, while transformation with pYCTEF alone did not reduce the resistance of the *sky1* Δ cells at all (Fig. 1A). Overexpression from the high-copy plasmid pEG202-*SKY1* (*SKY1* under the constitutive alcohol dehydrogenase promoter) also showed full complementation of the cisplatin resistance phenotype, whereas cells transformed with the empty vector alone were still resistant (Fig. 1B). Notably, the pEG202-*SKY1* transformants were markedly more sensitive to cisplatin than empty vector-transformed *SKY1*⁺ cells (Fig. 1B). These data strongly affirm that the cisplatin resistance, which was originally observed in the transposon-derived strains, was linked to disruption of the *SKY1* gene, and suggest that overexpression of *SKY1* makes yeast cells hypersensitive to cisplatin.

To rule out the possibility that NER deficiency would be functionally involved in the cisplatin resistance phenotype of *sky1* Δ cells, we used two independent, NER-proficient backgrounds. Because *rad52* Δ cells are about as sensitive to cisplatin as *rad4* Δ cells (McA'Nulty and Lippard, 1996), the *RAD52* gene involved in recombinational repair was first disrupted from NER-proficient *sky1* Δ cells by one-step gene replacement. The cisplatin sensitivity profile of the resulting *rad52* Δ *sky1* Δ mutants was then determined, compared with that of *rad52* Δ *SKY1*⁺ cells (Fig. 1C). *S. cerevisiae* *rad52* Δ *sky1* Δ cells were 3- to 4-fold more cisplatin-resistant than *rad52* Δ *SKY1*⁺ cells, which is similar to the effect of *SKY1* disruption in *rad4* Δ backgrounds. Second, the *SKY1* gene was disrupted in NER- and recombinational repair-proficient wild-type cells, which display a relatively flat dose-response curve compared with cisplatin. Strain W303-*sky1* Δ was generated by one-step gene replacement, and monitored for cisplatin resistance. Replacement of *SKY1* by a disrupted copy also made these wild-type cells resistant to cisplatin (Fig. 1D), with a striking similarity in resistance level: *S. cerevisiae* W303-*sky1* Δ cells were 3- to 4-fold cisplatin-resistant compared with isogenic MGSC186 *SKY1*⁺ cells. Taken together, these data show that *SKY1* disruption-derived cisplatin resistance is independent of the *rad4* Δ genotype. Notably, irrespective of their backgrounds, all replacement-derived *sky1* Δ strains displayed normal growth characteristics (data not shown).

Cross-Resistance of Yeast Cells Containing a Disrupted *SKY1* Gene. To determine the specificity of the resistance phenotype and to learn more about the underlying mechanisms, we assayed *sky1* Δ mutants for cross-resistance to other cytotoxic agents. Colony formation assays were performed with a range of different classes of chemicals (mostly anticancer drugs), including cisplatin analogs (carboplatin and oxaliplatin), heavy metals (cadmium chloride and sodium arsenite), classical alkylating agents [busulfan, streptozotocin, *N*-nitroso-*N*-methylurea (NMU), and procabazine], a bioreductive alkylating agent (mitomycin C), antimetabolites (6-mercaptopurine and 5-fluorouracil), a topoisomerase I inhibitor (camptothecin), topoisomerase II inhibitors (doxorubicin, daunorubicin, and etoposide), and an antibiotic (oligomycin). In addition, possible cross-resistance to DNA damaging agents (ionizing radiation and UV light) was monitored.

Disruption of *SKY1* did not only confer resistance to cisplatin, but also to the cisplatin analog carboplatin (Table 1; Fig. 2). Furthermore, the inactivation of *SKY1* conferred resistance to the anthracycline drugs doxorubicin and daunorubicin (Table 1; Fig. 2), which are thought to inhibit topoisomerase II through DNA intercalation (Pratt et al., 1994). However, sensitivity toward the cisplatin analog oxaliplatin and another topoisomerase II inhibitor, etoposide, was not changed. In addition, we did not observe any change in sensitivity toward sodium arsenite, busulfan, streptozotocin, NMU, procabazine, mitomycin C, 6-mercaptopurine, camptothecin, oligomycin, ionizing radiation, or UV light. Surprisingly, *sky1* Δ mutants were hypersensitive toward cadmium chloride and 5-fluorouracil. In Fig. 2, representative examples of compound concentration versus colony formation are given (i.e., survival curves for carboplatin, oxaliplatin, doxorubicin, cadmium chloride, 5-fluorouracil, and NMU are shown).

In eukaryotic cells, including yeast, resistance to anthracyclines might be caused by emergence of a multidrug resistance (MDR)/pleiotropic drug resistance (PDR) phenotype (Kolaczowska and Goffeau, 1999), characterized by overexpression of a drug efflux pump the activity of which can be blocked by a variety of modulators, including verapamil (Hogue et al., 1999). To rule out the possible involvement of an MDR/PDR phenotype in *sky1* Δ yeast, the cells were incubated with a concentration range of doxorubicin in the presence of a fixed dose of verapamil (1.7 mM) that had previously been shown to be effective in blocking an MDR phenotype in yeast (Hogue et al., 1999), and growth was determined in a semiquantitative spot assay. The dose of verapamil used did not affect cell growth by itself, neither did it have any effect on doxorubicin resistance in *sky1* Δ yeast cells (Fig. 3).

Platinum Accumulation and DNA Platination are Unaffected in Yeast *sky1* Δ Mutants. Recently, it was demonstrated that disruption of the *SKY1* gene results in a dramatically reduced uptake of the polyamines spermine, spermidine, and their precursor, putrescine, corresponding with tolerance to toxic levels of spermine (Erez and Kahana, 2001). To determine whether, by analogy, the cisplatin resistance phenotype of *sky1* Δ mutant cells was linked to im-

TABLE 1

Effect of *SKY1* disruption on yeast sensitivity to cytotoxic agents

Sensitivity was monitored by colony formation assays in *S. cerevisiae* *sky1* Δ cells (i.e., cells from which the *SKY1* gene had been disrupted) versus isogenic *SKY1*⁺ cells. For each agent, these assays were performed at least three times, and consistent results were obtained each time. Resistance or hypersensitivity factors were calculated from the IC₅₀ values as estimated from Figs. 1 and 2. Different classes of chemicals are indicated by footnotes.

Resistance	Cisplatin ^a (4-fold)	Carboplatin ^a (2-fold)
Hypersensitivity	Doxorubicin ^b (2-fold)	Daunorubicin ^b
	Cadmium chloride ^c (5-fold)	5-Fluorouracil ^d (3-fold)
No change	Oxaliplatin ^a	Etoposide ^b
	Sodium arsenite ^c	6-Mercaptopurine ^d
	Busulfan ^e	Streptozotocin ^e
	NMU ^e	Procabazine ^e
	Mitomycin C ^e	Camptothecin
	Oligomycin	
	Ionizing radiation	UV light

^a Platinum analogs.

^b Topoisomerase II inhibitors.

^c Heavy metals.

^d Antimetabolites.

^e Alkylating agents.

paired drug accumulation or platination, cellular platinum content and platinum-DNA adduct formation were monitored by AAS (Burger et al., 2000) and a postlabeling assay (Pluim et al., 1999), respectively. A clear dose-effect relationship between cisplatin exposure and both accumulation and platination was found. However, *S. cerevisiae sky1Δ* cells did

not seem to display decreased platinum accumulation compared with *SKY1*⁺ cells (data not shown). Furthermore, *S. cerevisiae* MGSC-*sky1Δ* cells did not show a decrease in platination compared with the original MGSC131 *SKY1*⁺ strain (Fig. 4). Therefore, the observed cisplatin resistance can probably not be attributed to major alterations in specific cell wall components, changes in plasma membrane permeability, impaired nuclear drug import or reduced formation of cytotoxic intrastrand platinum-DNA adducts (Pratt et al., 1994; Johnson et al., 1998).

Doxorubicin Accumulation Is Unaffected in Yeast *sky1Δ* Mutants. To reveal whether the doxorubicin resistance phenotype of *S. cerevisiae sky1Δ* mutants was associated with impaired drug accumulation, cellular doxorubicin content was determined by HPLC (de Bruijn et al., 1999). Analogous to the platinum accumulation and platination experiments, a clear dose-effect relationship between doxorubicin exposure and accumulation was found. Yet, likewise, MGSC-*sky1Δ* cells did not show a decrease in cellular doxorubicin content compared with the original *SKY1*⁺ strain (Fig. 5); neither did we see any effect of verapamil on doxorubicin accumulation in *sky1Δ* or *SKY1*⁺ cells (data not shown). So, in parallel to the situation for cisplatin, the observed doxorubicin resistance is most probably unrelated to decreased drug import or increased drug export.

Yeast *sky1Δ* Mutants Display a Mutator Phenotype. As changes in intracellular drug accumulation do not seem to play a role in the observed cisplatin and doxorubicin resistance phenotype, alternative mechanisms might explain our data. MMR deficiencies have previously been shown to correspond to resistance to both cisplatin and carboplatin, but not oxaliplatin, in cultured colon, endometrial, and ovarian cancer cells (Fink et al., 1996; Vaisman et al., 1998). Furthermore, it has been demonstrated that cisplatin and doxorubicin resistance are associated with MMR deficiency in an ovarian tumor cell line (Drummond et al., 1996), and that

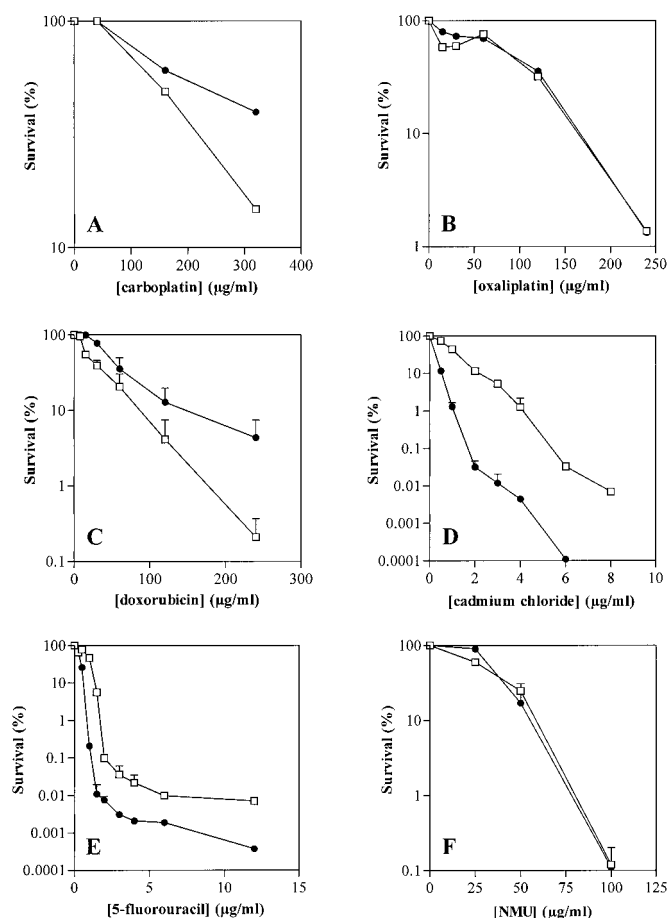


Fig. 2. Sensitivity to cytotoxic compounds of yeast *sky1Δ* versus *SKY1*⁺ cells. A to F, sensitivity profiles of *S. cerevisiae* MGSC131 *SKY1*⁺ cells (□) and isogenic MGSC-*sky1Δ* cells (●) toward the agent indicated on the x-axis. Percentage survival (colony formation) at each compound concentration is expressed relative to untreated control cells (100%). Means of typical experiments performed in triplicate are shown, with bars representing S.D. Bars are sometimes masked by the data point symbols. Please note that the ordinates of the graphs are in different ranges.

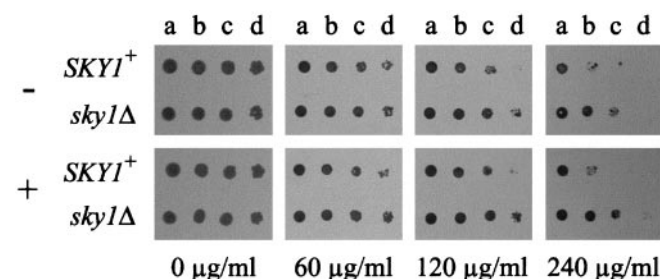


Fig. 3. Effect of MDR modulator verapamil on doxorubicin sensitivity of *S. cerevisiae sky1Δ* versus *SKY1*⁺ cells. Yeast MGSC131 *SKY1*⁺ and isogenic MGSC-*sky1Δ* cells were tested for their relative ability to grow on selective medium plates containing the indicated doxorubicin concentrations (0, 60, 120 or 240 μg/ml) in the absence (–, top row) or presence (+, bottom row) of 1.7 mM verapamil by spotting aliquots of 10⁵ (a), 10⁴ (b), 10³ (c) and 10² (d) cells, and incubating at 30°C for 4 days.

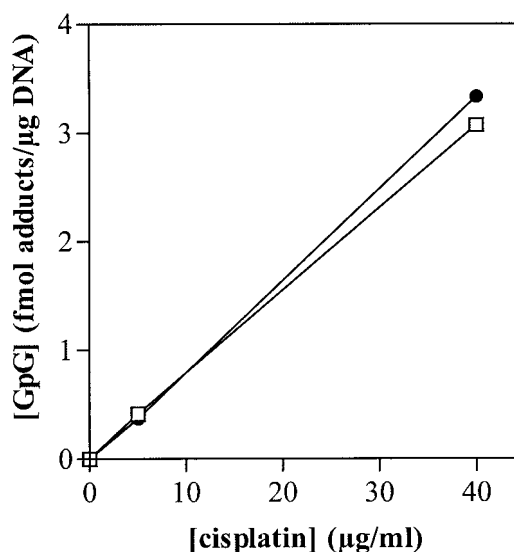


Fig. 4. DNA platination in yeast *sky1Δ* versus *SKY1*⁺ cells. *S. cerevisiae* MGSC131 *SKY1*⁺ cells (□) and isogenic MGSC-*sky1Δ* cells (●) were incubated with various concentrations of cisplatin, and DNA platination was measured by postlabeling. Data from a typical experiment (of three performed in duplicate) are shown. The ApG adduct levels, which were much lower than the GpG levels shown here, did not clearly differ between the yeast strains either.

loss of MMR renders yeast cells resistant to cisplatin, carboplatin, and doxorubicin (Durant et al., 1999). It could thus be proposed that reduced MMR might contribute to drug resistance in the *sky1Δ* yeast cells.

Because MMR defects are generally accompanied by a so-called mutator phenotype (Branch et al., 1995), we explored whether *sky1Δ* cells displayed an enhanced rate of spontaneous mutation compared with *SKY1*-proficient cells. The rate of forward mutation at the *URA3* locus was determined for MGSC-*sky1Δ* cells and isogenic MGSC284 *SKY1*⁺ cells. As shown in Fig. 6, there was a 2.4-fold increase of the mutation rate per replication in the *sky1Δ* strain (rate = 7.4×10^{-7}), compared with the *SKY1*⁺ strain (rate = 3.1×10^{-7}). This indicates that, indeed, disruption of the *S. cerevisiae SKY1* gene induces a mutator phenotype.

Discussion

We have used the budding yeast *Saccharomyces cerevisiae* to search for novel genes whose functional abrogation confers cellular resistance to the commonly applied anticancer agent cisplatin, and found that a 4-fold cisplatin resistance was linked to disruption of the *SKY1* gene (Schenk et al., 2001). Because constitutive expression of *SKY1* from a high-copy vector made *sky1Δ* yeast cells markedly more sensitive to cisplatin compared with a *SKY1*⁺ control strain, *SKY1* may be a cisplatin sensitivity gene that is actively involved in the pathway of cisplatin-induced cell kill. One of the physiological functions of Sky1p could actually be to down-regulate RNA processing (leading to growth inhibition or even cell death) upon its translocation from the cytoplasm to the nucleus in response to specific events (Siebel et al., 1999), including treatment with antiproliferative drugs.

In cross-resistance studies, we exclusively found resistance of *sky1Δ* yeast cells to cisplatin, carboplatin, and the anthracyclines doxorubicin and daunorubicin. Apparently, the re-

sistance is not associated with covalent DNA binding per se, because *sky1Δ* cells did not show cross-resistance to other alkylators, such as the alkane sulfonate busulfan, the nitrosourea streptozotocin and NMU, and the methylating agent procarbazine. The anthracyclines doxorubicin and daunorubicin (drugs that interact with DNA by intercalation), as well as the epipodophyllotoxin etoposide, are thought to have the enzyme topoisomerase II as their main intracellular target (Pratt et al., 1994). The lack of cross-resistance to etoposide strongly suggests that topoisomerase II is not involved in the anthracycline-resistant phenotype of *sky1Δ* cells. Therefore, another consequence of DNA intercalation might be of vital importance here. Another set of drugs that we compared for changes in the capacity to inhibit yeast colony formation was the hypoxanthine analog and purine antagonist 6-mercaptopurine versus the pyrimidine antagonist 5-fluorouracil. Although no change in the response to 6-mercaptopurine was observed, hypersensitivity to 5-fluorouracil was evident in *sky1Δ* mutants.

The doxorubicin resistance phenotype of yeast *sky1Δ* mutant cells does not seem to be linked to impaired doxorubicin accumulation. Therefore, a role for the *S. cerevisiae* PDR network, which comprises the functional P-glycoprotein homolog Pdr5p and the MRP homolog Yor1p (Kolaczowska and Goffeau, 1999), seems unlikely. Our finding that the sensitivity of *sky1Δ* cells toward oligomycin was unaltered, is in line with this, as overexpression of Yor1p has previously been associated with resistance to this drug (Katzmann et al., 1995). The yeast activator protein-1-like factor network comprising the Ycf1p detoxification pump for glutathione conjugates, which is essential to cadmium tolerance (Kolaczowska and Goffeau, 1999), does probably not play a role in the observed cisplatin or doxorubicin resistance either, because activation of this pump would also lead to reduced drug accumulation. Yet, this network may be affected in *sky1Δ* cells, rendering them hypersensitive to cadmium chloride. Although heavy metals are generally thought to share cellular pathways modulating their toxicity, it has previously been shown that cisplatin-resistant cells are not necessarily cross-resistant to cadmium, and, vice versa, cadmium-hypersensitive cells are not automatically hypersensitive to cispla-

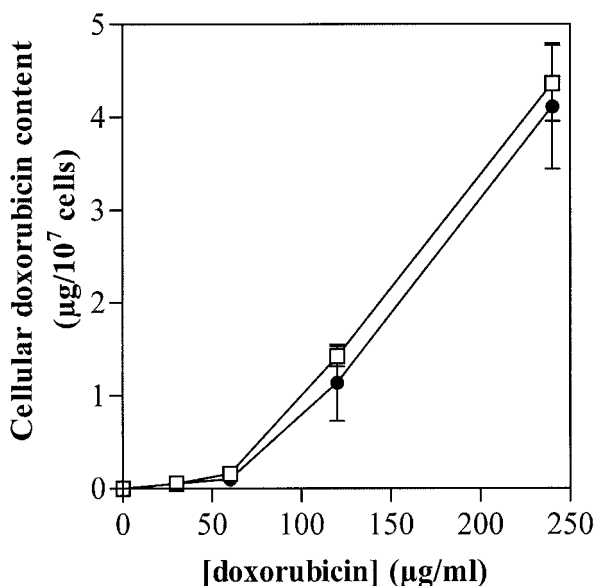


Fig. 5. Doxorubicin accumulation in yeast *sky1Δ* versus *SKY1*⁺ cells. *S. cerevisiae* MGSC131 *SKY1*⁺ cells (□) and isogenic MGSC-*sky1Δ* cells (●) were incubated with various concentrations of doxorubicin, and cellular drug content was measured by HPLC. Means of a typical experiment performed in duplicate are shown, with bars representing S.D. Bars are sometimes masked by the data point symbols.

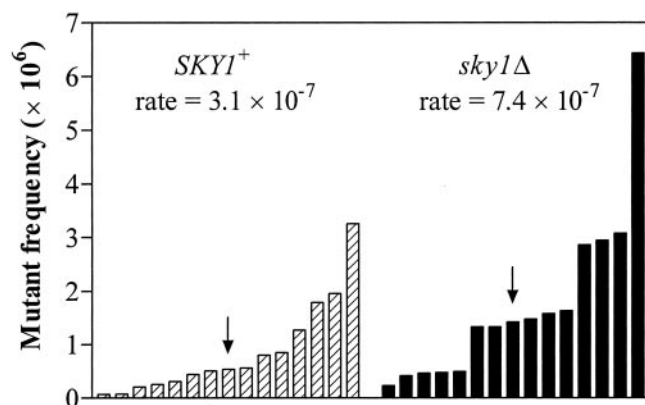


Fig. 6. Spontaneous mutation rates in yeast *sky1Δ* versus *SKY1*⁺ cells. Mutant frequencies at the *URA3* locus were determined in 15 individual parallel cultures of either *S. cerevisiae* MGSC284 *SKY1*⁺ cells (▨) or isogenic MGSC-*sky1Δ* cells (■). For each strain, bars are grouped according to the data obtained, with frequencies increasing from left to right. The mutation rates per replication were calculated from the median mutant frequencies indicated by vertical arrows.

tin. In parallel to the findings presented here, a cisplatin-resistant murine leukemia cell line was found to be hypersensitive to cadmium (Farnworth et al., 1990). Conversely, a cadmium-hypersensitive *Schizosaccharomyces pombe* mutant was demonstrated to be as tolerant to cisplatin as the corresponding wild-type strain (Perego et al., 1997). Interestingly, Erez and Kahana (2001) have recently shown that *S. cerevisiae sky1Δ* cells exhibit increased tolerance to high concentrations of some inorganic ions (0.2 M lithium chloride or 1.2 M sodium chloride), but increased sensitivity to osmotic shock at 1.5 M potassium chloride or 1.5 M sorbitol.

Summarizing our present cytotoxicity data, obtained comparing yeast *sky1Δ* cells to isogenic *SKY1*⁺ cells, the most striking pattern was that of resistance to cisplatin, carboplatin and doxorubicin, unaltered sensitivity to oxaliplatin, and hypersensitivity to 5-fluorouracil. As mentioned earlier, loss of MMR has previously been associated with resistance to cisplatin, carboplatin and doxorubicin, but not oxaliplatin, in mammalian cells as well as in yeast (Drummond et al., 1996; Fink et al., 1996; Vaisman et al., 1998; Durant et al., 1999). In drug-sensitive cells, binding of MMR proteins to cisplatin- or carboplatin-derived DNA adducts may directly or indirectly activate signal transduction pathways involved in cell kill. MMR proteins seem to function as detectors of platinum-DNA 1,2-intrastrand GpG cross-links induced by cisplatin and carboplatin (Duckett et al., 1996; Fink et al., 1996) and probably propagate signals that contribute to activation of programmed cell death (Toft et al., 1999). Recently, it has been suggested that doxorubicin inhibits the correction of DNA mismatches in vitro by preventing MutS α -mediated mismatch recognition through reversible DNA intercalation, which might at last link doxorubicin to MMR-dependent drug resistance mechanistically (Larson and Drummond, 2001). Interestingly, recent findings by Hemminki et al. (2000) imply that loss of MMR might also contribute to 5-fluorouracil hypersensitivity. It was shown that, after 5-fluorouracil-based therapy, the prognosis of colorectal cancers characterized by microsatellite instability (a hallmark of an MMR defect) is significantly better than that of patients without microsatellite instability, suggesting that MMR-deficient tumors could be hypersensitive to 5-fluorouracil (Hemminki et al., 2000). Given our drug sensitivity data and the finding that *S. cerevisiae sky1Δ* cells also display a mutator phenotype (another hallmark of an MMR defect), the MMR system may thus be impaired in these yeast cells.

In human cancer cells, MMR deficiency has also been associated with tolerance to DNA methylation damage inflicted by agents such as NMU, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, temozolomide, and procarbazine (Branch et al., 1995; Friedman et al., 1997). In *S. cerevisiae*, however, it has been shown that mutations in MMR genes (except *MSH5*) do not render cells more tolerant to methylation-induced kill (Bawa and Xiao, 1997), in line with our present data. This hints at alternative mechanisms involved in methylation-induced killing, and indicates that there may be a discrepancy between yeast and mammalian cells with respect to tolerance to methylating agents.

Durant et al. (1999) recently demonstrated that, whereas disruption of several MMR genes, including *MLH1*, *MLH2*, and *MSH2*, led to cisplatin, carboplatin and doxorubicin resistance in *S. cerevisiae RAD52*⁺ cells, inactivation of *MLH1*,

MLH2, or *MSH2* in a yeast *rad52Δ* strain did not result in cisplatin or carboplatin resistance. Because we show that *rad52Δ sky1Δ* cells are 3- to 4-fold cisplatin-resistant compared with *rad52Δ SKY1*⁺ cells (which is a resistance level similar to that observed in a *rad4Δ* or wild-type background), the cisplatin resistance observed in our present study can probably not be straightforwardly explained by deficiencies in MMR proteins Mlh1p, Mlh2p, or Msh2p. Nevertheless, deregulation of other MMR components, such as Msh3p or Msh6p, may have an effect on drug sensitivity in *S. cerevisiae rad52Δ* backgrounds and could thus be involved in the cisplatin-resistant phenotype of *sky1Δ* cells.

However, cisplatin resistance in combination with a mutator phenotype might, for instance, still (partly) result from up-regulation of functional homologs to DNA polymerase β , which is involved in base excision repair. It has recently been demonstrated that elevated activity of DNA polymerase β , corresponding to enhanced translesion synthesis across platinated DNA cross-links, occurs in cisplatin-resistant human ovarian carcinoma cells; at the same time, DNA polymerase β is one of the most inaccurate DNA synthesizing enzymes conferring genetic instability when up-regulated in such cells (Bergoglio et al., 2001). Alternatively, an up-regulation of the Rev3p (DNA polymerase ζ) pathway involved in error-prone damage tolerance could possibly (partly) underlie both cisplatin resistance and the mutator phenotype of *sky1Δ* cells, as *S. cerevisiae rev3Δ* cells are hypersensitive to cisplatin (Simon et al., 2000) and possess an antimutator phenotype (Roche et al., 1994). This alternative hypothesis may, however, not account for other aspects of the *sky1Δ* phenotype, for instance doxorubicin resistance, because *REV3* disruption seems to cause doxorubicin resistance instead of hypersensitivity (Simon et al., 2000).

Anyhow, the exact pathway through which Sky1p contributes to the cytotoxic action of cisplatin, carboplatin, and doxorubicin in yeast remains to be resolved in more detail. Heterologous yeast complementation experiments, employing wild-type versus kinase dominant-negative human SRPK1, strongly suggest that the protein kinase function of SR-protein-specific kinases is essential to their involvement in the cytotoxicity of cisplatin (Schenk et al., 2001). Although Sky1p may fulfill its role in increasing sensitivity to lithium chloride via the protein phosphatase Ppz1p comprising SR segments at its NH₂-terminal region, it has not yet been established whether Ppz1p is indeed phosphorylated by Sky1p (Erez and Kahana, 2001). The *S. cerevisiae* SR-protein Npl3p (nuclear protein localization) is phosphorylated by Sky1p both in vitro and in vivo (Siebel et al., 1999), and shuttles between the cytoplasm and the nucleus (where it resides primarily at steady state) to deliver proteins and/or mRNA. Phosphorylation of Npl3p and mammalian SR-proteins modulates protein-protein and protein-RNA interactions. In *sky1Δ* cells, Npl3p is mislocalized to the cytoplasm because of impaired interaction between Npl3p and its nuclear import receptor and, thus, decreased nuclear import (Yun and Fu, 2000). This might implicate that Npl3p-mediated nuclear import of (accessory) components involved in drug-induced cell kill would also be decreased in yeast *sky1Δ* cells, leading to deregulation of processes influencing cytotoxicity. Of course, Sky1p may be a key player in other pathways determining drug sensitivity as well and, therefore, additional mechanisms may contribute to the resistance

and hypersensitivity phenotype observed in *S. cerevisiae* *sky1Δ* cells.

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