Creation of a Hyperpermeable Yeast Strain to Genotoxic Agents through Combined Inactivation of PDR and CWP Genes

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We previously established a genotoxicity detection system based on the transcriptional response of the yeast RNR3 gene to DNA damage. In order to further improve its sensitivity to genotoxicants, we have attempted to increase cell permeability by removing cell wall mannoproteins (CWPs). Here, we report that selected deletion of pleiotropic drug resistance (PDR) genes encoding membrane efflux transporters also enhanced cellular sensitivity to treatment by various genotoxic agents. Furthermore, we have validated our hypothesis that PDR and CWP protect cells through different mechanisms by demonstrating that simultaneous inactivation of the above two pathways resulted in a synergistic enhancement of assay sensitivity as measured by RNR3-lacZ expression and that this effect is at the cell permeability level. The quadruple mutation results in RNR3-lacZ assay sensitivity to tested chemicals that apparently surpasses the industry standard Ames test. We argue that this hyperpermeable yeast mutant strain would be suitable for other chemical-based genotoxic assays.

Key Words: Saccharomyces cerevisiae; RNR3-lacZ; genotoxicity test; permeability; ABC transporters; cell wall.

Development of microorganism-based, environmentally safe, and highly sensitive genotoxic testing systems remains an intensive research area in the field of toxicology. The budding yeast Saccharomyces cerevisiae is an ideal choice because this species has been used in a variety of fermentation industries, including making bread, beer, and wine, and is hence safe for the environment and public health. Furthermore, budding yeast is the best-studied model lower eukaryotic microorganism, which is more comparable to human cells than bacteria. Surprisingly, despite the presence of a very strong budding yeast research community and the fact that this species was the first eukaryotic organism whose genomic sequence was determined, little attention has been paid to genetic factors that influence the sensitivity of budding yeast to toxic chemicals.

We previously developed a genotoxicity testing system based on the transcriptional response of the RNR3-lacZ reporter gene to a broad range of DNA-damaging agents in budding yeast (Jia et al., 2002). Although the sensitivity of this method to detect genotoxicants is comparable to the bacterial chromotest, which is also based on transcriptional response to DNA damage, in many cases, the RNR3-lacZ testing system is not as sensitive to the best-studied model and industrial standard Ames test. The Ames test sensitivity to detect certain types of carcinogenic chemicals was significantly improved through several genetic modifications, including the removal of the lipopolysaccharide coat from Salmonella typhimurium to make cells more permeable to higher–molecular weight compounds. We reasoned that yeast genes involved in cell wall and membrane biosynthesis could be modified genetically to alter cellular permeability and improve the toxicity test in yeast. Indeed, deletion of both CWP1 and CWP2 genes encoding cell wall mannoproteins (CWPs) markedly increased cell wall permeability and enhanced the RNR3-lacZ assay sensitivity, particularly to higher–molecular weight compounds (Zhang et al., 2008). In contrast, deletion of some nonessential membrane components did not significantly affect the RNR3-lacZ sensitivity to the testing chemicals (Zhang et al., 2008 and data not shown), which allowed us to conclude that the yeast CWPs constitute a major barrier to genotoxic agents.

In addition to the physical barrier provided by cell wall and membrane, budding yeast also possesses a dynamic biochemical defense system against xenobiotics called pleiotropic drug response or pleiotropic drug resistance (PDR) (Balzi and Goffeau, 1995). PDR is mediated by a network of ATP-binding cassette (ABC) transporter proteins that constitute active efflux pumps for a broad spectrum of unrelated chemicals. The PDR network currently consists of 10 transcription factors regulating about 70 different target genes, of which 22 genes have been identified with putative ABC transporter activity (Emst et al., 2005; Moye-Rowley, 2003). The three major ABC transporter genes in yeast are PDR5 (Golin et al., 2007), YOR1 (yeast oligomycin resistance) (Cui et al., 1996), and SNQ2 (sensitivity to 4-nitroquinoline-N-oxide) (Servos et al., 1993). PDR5, YOR1, and SNQ2 have...
substrate specificities as well as overlapping functions for some chemicals (Kolaczkowski et al., 1996, 1998). In addition, PDR1 and PDR3 encode two zinc finger transcription factors that are required for the activation of most ABC transporter genes (Katzmann et al., 1994; Mannun et al., 2002). Pdr1 and Pdr3 can form either a homodimer or a heterodimer, which may play a role in target gene specificity. Indeed, deletion of PDR1 or PDR3 results in differential drug tolerance; nevertheless, loss of both genes aggravates drug hypersensitivity due to impaired drug transporters (Rogers et al., 2001).

In this report, we systematically examined the effects of the inactivation of major yeast ABC transporter genes on the RNR3-lacZ genotoxicity test with selected chemicals. We then hypothesized that the enhanced sensitivity by deleting PDR genes is mechanistically distinct from that of deleting CWP genes and demonstrated that indeed the combined inactivation of both cellular defense systems resulted in an unprecedented level of sensitivity to detecting certain toxic chemicals.

MATERIALS AND METHODS

Yeasts strains, cell culture, and transformation. Haploid S. cerevisiae yeast strains used throughout this study are summarized in Supplementary table 1. FY1679-28C and its isogenic derivatives were received from either Dr. A. Delahodde (Centre National de la Recherche Scientifique, Paris, France) or Dr. A. Kolaczkowska (University of Wroclaw, Wroclaw, Poland). The pdr5 snq2 yor1 triple mutant grew slowly in the synthetic selective medium lacking uracil (data not shown) and hence was not further analyzed.

Yeast cells were grown at 30°C in YPD (Sherman et al., 1983). Plasmid DNA was transformed into yeast cells by a modified lithium acetate protocol (Hill et al., 1991) and selected on minimal SD medium (Sherman et al., 1983). Transformants were streaked on a fresh selective plate before being utilized for further analysis.

Plasmids and mutant strain construction. Plasmid pZZ2 (Zhou and Elledge, 1992) was obtained from Dr. S. Elledge (Harvard University, Boston, MA) and utilized for the RNR3-lacZ test as previously described (Jia and Xiao, 2003; Jia et al., 2002).

To create the cwp1::hisG mutant, plasmid pGEM-CWP1 containing a 0.72-kb CWP1 open reading frame plus 0.7-kb flanking region (Zhang et al., 2008) was used to delete a 0.62-kb Sty1 fragment within the CWP1 ORF, which was replaced by a BamHI linker to form pcwp1AB. A 3.8-kb BamHI-By HindIII fragment from pNKY51 containing the hisG-URA3-hisG cassette (Alani et al., 1987) was then cloned into the BamHI site of pcwp1AB to form pcwp1::Huh. The cwp1::hisG-URA3-hisG cassette was released by XhoI-SphI digestion prior to yeast transformation. The cwp1 deletion mutant was allowed to grow nonselectively overnight and then selected on a plate containing 5-fluoro-orotic acid as described (Boeke et al., 1984) to obtain the cwp1::hisG derivative.

To construct the cwp2::HS3 disruption plasmid, a 0.5-kb EcoRI-BamHI fragment containing the 5′ flanking region and a 0.3-kb BamHI-SphI fragment containing the 3′ flanking region of CWP2 ORF was sequentially cloned into pGEM-T (Promega, Madison, WI) to form pcwp2AB. The 1.16-kb BamHI fragment from YDP-H (Berben et al., 1991) containing the HS3 gene was then cloned into the BamHI site of pcwp2AB to form pcwp2::HS3. The cwp2::HS3 cassette was released by EcoRI-SphI digestion prior to transformation.

Test chemicals. Genotoxic chemicals used in this study include methyl methanesulfonate (MMS), 4-nitroquinolino-N-oxide (4-NNO), phleomycin, which have been previously described in Zhang et al. (2008), and the two anticancer agents, chlorambucil (304 Da) and cisplatin (300 Da). MMS (110 Da) represents a low-molecular weight mutagen, 4-NNO (190 Da) represents a medium–molecular weight mutagen, while phleomycin (1526 Da) is considered to be a bulky molecule. In addition, L-canavanine (274 Da) and tetracycline (444 Da) were used to represent nongenotoxic agents. All the above chemicals were purchased from Sigma-Aldrich (St Louis, MO).

DNA damage treatment and β-galactosidase assay. The β-galactosidase (β-gal) assay was performed as described previously (Jia and Xiao, 2004; Xiao et al., 1993). Briefly, 0.5 ml of overnight yeast culture was used to inoculate 2.5 ml of fresh SD selective medium (Supplemental materials and methods) and incubation was continued for another 2 h. At this point, chemicals were added at the concentration indicated and cells were incubated for another 4 h. Conditions for ultraviolet (UV) irradiation have been previously described (Jia et al., 2002). After the incubation, 1 ml of the above unsynchronized log-phase cell suspension was used to determine cell titer by measuring optical density at 600 nm, and the remaining 2 ml yeast cells were precipitated by centrifugation, washed twice with sterile distilled water, and resuspended in a Z buffer (60 mM Na2HPO4, 7 mM H2O, 40 mM NaH2PO4, 4 mM H2O, 10 mM KCl, 1 mM MgSO4, 7 mM H2O, and 40 mM β-mercaptoethanol, pH 7.0) for the β-gal assay using orthochloro-nitrophenyl-β-galactoside as the colorimetric substrate. The β-gal activity is expressed in Miller units (Guarente, 1983).

Toxicity test. Cell survival rates were determined as previously described (Jia et al., 2002). At the end of incubation and prior to the β-gal assay, untreated and treated cells were collected by centrifugation, diluted, and plated on YPD in duplicate. The plates were incubated at 30°C for 3 days, and the number of colonies was counted. The toxic effect is expressed as a percentage of colonies from treated samples versus untreated samples.

Statistical analysis. All the results presented in this report were an average of at least three independent experiments and expressed as mean ± SD. Statistical significance between different strains was determined using a two-tailed pair Student’s t-test. A p value of less than 0.01 was considered statistically significant and less than 0.0001 highly significant.

RESULTS

Deletion of PDR Genes Enhances the RNR3-lacZ Sensitivity

To ask whether the inactivation of ABC transporters affects cell permeability to DNA-damaging agents, three representative chemicals, MMS, 4-NNO, and phleomycin, were selected in this study. They are all genotoxic and carcinogenic agents with different molecular size, chemical structure, and mechanism of action. The RNR3-lacZ reporter gene assay (Jia et al., 2002) was primarily employed to assess effects of gene mutations on the cellular responses to test chemicals, although the cell survival after chemical treatment can also reflect the toxicity.

As shown in Figure 1, compared with wild-type cells, deletion of either PDR5 or YOR1 resulted in indistinguishable responses to all three drugs tested with respect to both RNR3-lacZ induction and cytotoxicity. In contrast, deletion of SNQ2 resulted in a significant enhancement of RNR3-lacZ induction by 4-NQO (Fig. 1A) and moderate increases in phleomycin (Fig. 1B) and MMS (Fig. 1C) induction. Specifically, after treatment with 0.5 μg/ml 4-NQO for 4 h, snq2 mutant cells displayed almost 4-fold induction compared with less than 1.5-fold induction in the wild type or other two single mutants (Fig. 1A). In addition, the maximum induction was increased from 6-fold in the wild type to 11-fold in the snq2 mutant. Similarly, the snq2 mutant appears to be slightly more sensitive...
to killing by the three DNA-damaging agents examined (Figs. 1D–F). The specificity of Snq2 for 4-NQO is not surprising as the snq2 mutant was initially identified for its enhanced sensitivity to 4-NQO (Servos et al., 1993).

The ABC transporters often confer complex overlapping substrate specificities. It has been reported that if one PDR gene is deleted, the remaining transporters may compensate, whereas if all three genes are inactivated, the drug resistance will be impaired (Rogers et al., 2001). These overlapping functions prompted us to ask whether simultaneous deletion of two or more PDR genes will result in an additive or synergistic effect on drug permeability. As shown in Figure 2, the pdr5 snq2 double mutant displayed an increased sensitivity to 4-NQO–induced or phleomycin-induced RNR3-lacZ expression (Figs. 2A and 2B). For example, at a 4-NQO concentration of 0.25 μg/ml, the double mutant displayed a fourfold induction, whereas the induction in the wild type and other single mutant was barely detectable (Fig. 2A). Similarly, a phleomycin concentration of 5 μg/ml, the pdr5 snq2 double mutant displayed more than twofold induction, whereas the induction in the wild type and other single mutant was barely detectable (Fig. 2B). As for MMS, the double mutation also enhanced the RNR3-lacZ induction, albeit to a lesser extent (Fig. 2C) than induction by 4-NQO or phleomycin. Consistent with the RNR3-lacZ induction, the pdr5 snq2 double mutant also became more sensitive to killing by 4-NQO (Fig. 2D) or phleomycin (Fig. 2E) compared with wild type and the snq2 single mutant. These observations indicate that although deletion of PDR5 alone has no effect on membrane permeability to chemicals like 4-NQO and phleomycin, in the absence of the major transporter Snq2, Pdr5 plays a backup role in preventing entry of these toxic chemicals.

The other two double mutants (yor1 snq2 and pdr5 yor1) also showed increased sensitivity to 4-NQO compared with wild-type cells; however, the effect was not as obvious as the snq2 pdr5 double mutation. It is of interest to note that although deletion of YOR1 in the snq2 background did not further enhance its sensitivity to 4-NQO, the pdr5 yor1 double mutant displayed an enhanced sensitivity to 4-NQO (Figs. 2A and 2D) despite the fact that neither corresponding single mutation had a noticeable effect (Figs. 1A and 1D). In contrast, these two double mutants showed little enhancement of sensitivity to phleomycin (Figs. 2B and 2E) or MMS (Figs. 2C and 2F). Based on the above analyses and the observed slow growth phenotype of the triple mutant (data not shown), we chose the pdr5 snq2 double mutant to represent ABC transporter–deficient strain for the subsequent investigation.

FIG. 1. Sensitivity of pdr single mutants to DNA-damaging agents. (A–C) RNR3-lacZ assays; (D–F) cell killing experiments. (A and D) 4-NQO treatment, (B and E) phleomycin treatment, and (C and F) MMS treatment. Results are the average of at least three independent experiments with SDs; *p < 0.01. Yeast strains used: (●) FY1679-28C (wild type), (▲) FYMK-1/1 (pdr5-D1), (■) FYAK4 (yor1-1), and (◇) FYMK 23/2 (snq2-D1).
Deletion of PDR3, but Not PDR1, Results in a Compromised RNR3-lacZ Induction

Budding yeast contains at least five ABC transporter subfamilies and many of them confer drug resistance (Bauer et al., 1999). It would be technically challenging to investigate each individual ABC transporter mutation and then double or multiple mutations for their combined effects on various drug-induced toxicity. It is known that a large number of ABC transporter genes are under the control of two master transcriptional activators, Pdr1 and Pdr3, which confer distinct as well as overlapping functions (Bauer et al., 1999). Since most of the stress-induced ABC transporter gene expression is virtually abolished in the pdr1 pdr3 double mutant (Mahe et al., 1996), we decided to measure the RNR3-lacZ expression in the above double mutant instead of individual transporter gene mutants. To our surprise, the RNR3-lacZ induction by all three testing compounds was significantly reduced in the pdr1 pdr3 double mutant (Fig. 3). This phenomenon is reminiscent of our previous finding that Pdr3 is a positive regulator of two DNA damage–inducible genes, MAG1 and DD11, and that deletion of PDR3, but not PDR1, abolished the MMS-induced expression of these two genes (Zhu and Xiao, 2004). To ask whether RNR3-lacZ induction is also affected by PDR3 but not PDR1, we measured the RNR3-lacZ induction by the three test compounds in the pdr1 and pdr3 single mutants and compared with wild type and the double mutant. As shown in Figures 3A–C, in all cases, deletion of PDR1 alone has no effect on the RNR3-lacZ expression, whereas deletion of PDR3 has the same effect as that of the double mutation. Hence, it is apparent that only Pdr3 is involved in the transcriptional regulation of RNR3 and that inactivation of these PDR transactivators is unsuitable for the enhancement of the RNR3-lacZ sensitivity. Interestingly, while deletion of either PDR1 or PDR3 did not enhance cellular sensitivity to the testing chemicals, the simultaneous inactivation of both genes increased cellular toxicity of 4-NQO and phleomycin but not MMS (Figs. 3D–F).

Combined Deletion of CWP and PDR Genes Enhances the RNR3-lacZ Sensitivity

The above results demonstrate that inactivation of ABC transporters, especially the pdr5 snq2 double mutation, has a pronounced effect on cell permeability and hence enhances the sensitivity of the RNR3-lacZ genotoxicity testing. In a previous study, we reported that inactivation of CWP genes enhances the RNR3-lacZ sensitivity as well (Zhang et al., 2008). Since PDR and CWP appear to protect cells by different
mechanisms, we hypothesized that the combined inactivation of PDR and CWP genes would have a synergistic effect on the cellular sensitivity to certain toxic agents. To test this hypothesis, we made a cwp1 cwp2 pdr5 snq2 quadruple deletion strain to simultaneously disarm two major yeast protection mechanisms, namely cell wall and the efflux pumps.

The quadruple mutant displayed extreme sensitivity to test chemicals compared with either of the double mutants (Fig. 4). For example, a greater than twofold enhancement in the RNR3-lacZ induction can be observed in the quadruple mutant at the lowest 4-NQO dose tested (0.031 μg/ml), whereas the RNR3-lacZ induction was not detected in the cwp1 cwp2 double mutant by 0.125 μg/ml or in the snq2 pdr5 double mutant by 0.0675 μg/ml 4-NQO (Fig. 4A). It was also observed that the snq2 pdr5 double mutant is more sensitive to 4-NQO than the cwp1 cwp2 double mutant (p < 0.01). A linear regression analysis using low-dose data indicates that the quadruple mutant (y = 35.1x + 2.327) enhances the RNR3-lacZ sensitivity by 11.2-fold over wild type (y = 3.13x + 1.700) compared with wild type (y = 0.0274x + 1.276), by 6.13-fold over the cwp1 cwp2 double mutant (y = 5.72x + 1.273), and by 2.7-fold over the snq2 pdr5 double mutant (y = 13.2x + 1.125). The quadruple mutation also further enhances the cytotoxicity of the test compounds (p < 0.001), although the combined effect may not be as impressive as that of the RNR3-lacZ assay (Fig. 4D).

The quadruple mutant also displayed synergistic sensitivity to phleomycin-induced RNR3-lacZ expression (Fig. 4B). At a phleomycin concentration of 2.5 μg/ml, the quadruple mutant displayed a 2.7-fold induction, whereas the induction in the snq2 pdr5 and cwp1 cwp2 double mutants was barely detectable. After treatment with 10 μg/ml phleomycin, the snq2 pdr5 double mutant showed 2.4-fold induction and the cwp1 cwp2 double mutant showed 2.9-fold induction, while the quadruple mutant showed over 6-fold induction. A linear regression analysis indicates that the detection sensitivity was increased by 27.5-fold in the quadruple mutant (y = 0.753x + 1.700) compared with wild type (y = 0.0274x + 1.276), by 3.1-fold over the cwp1 cwp2 double mutant (y = 0.246x + 1.022), and by 4.1-fold over the snq2 pdr5 double mutant (y = 0.183x + 1.121). The quadruple mutant was also more sensitive to killing by phleomycin than by snq2 pdr5 and cwp1 cwp2 double mutants (p < 0.01), and the effect appears to be additive (Fig. 4E).

Even for small–molecular weight compounds like MMS, the quadruple mutant still displayed an increased sensitivity compared with the corresponding double mutants (p < 0.001). With both RNR3-lacZ induction and cell survival experiments, the effects of pdr and cwp mutations are additive (Figs. 4C and 4F). At an MMS concentration of 6.25 ppm, the
quadruple mutant displayed a twofold induction, whereas the induction in the snq2 pdr5 and cwp1 cwp2 double mutants was barely detectable (Fig. 4C). A linear regression analysis showed that the detection sensitivity was increased by twofold in the quadruple mutant \((y = 0.6x + 0.79)\) over wild-type cells \((y = 0.3x + 0.33)\) and the sensitivity in cwp1 cwp2 double mutant \((y = 0.42x + 0.29)\) is only slight higher than in the wild type. A similar additive effect was also observed for cell survival after MMS treatment (Fig. 4F).

Pdr and Cwp Mutations Enhance Sensitivity to Cancer Chemotherapeutic Agents

As an initial attempt to demonstrate the general applicability of the above genetic modifications, we examined the effects of cwp and pdr mutations on the cellular sensitivity to chlorambucil and cisplatin, which are DNA-damaging agents used as chemotherapeutic drugs. Chlorambucil is a bifunctional alkylating agent similar to nitrogen mustard and has been mainly used in the treatment of chronic lymphocytic leukemia (Bank, 1992). Cisplatin is a platinum-based chemotherapeutic agent used to treat various types of cancers by forming cisplatin-DNA adducts (Fichtinger-Schepman et al., 1985). It is noted that while each pdr or cwp double mutation barely enhanced sensitivity to chlorambucil (Fig. 5A) and has a moderate effect on cisplatin (Fig. 5B)-induced \(RNR3\)-lacZ expression, the effect of quadruple mutation was much more obvious. A linear regression analysis showed that the sensitivity to chlorambucil was increased by 16.6-fold in the quadruple mutant \((y = 0.46x + 0.7914)\) over wild type \((y = 0.02768x + 1.084)\), 6.2-fold over the cwp1 cwp2 double mutant \((y = 0.07432x + 0.8218)\), and 8.6-fold over the snq2 pdr5 double mutant \((y = 0.05353x + 0.8603)\). The cisplatin detection sensitivity was increased by 2.6-fold in the quadruple mutant \((y = 0.1666x + 2.601)\) over the wild type \((y = 0.06423x + 0.9265)\), 2.0-fold over the cwp1 cwp2 double mutant \((y = 0.08186x + 1.954)\), and 2.3-fold over the snq2 pdr5 double mutant \((y = 0.07176x + 2.2112)\). Compared to cwp or pdr double mutant, the quadruple mutant also displayed enhanced sensitivity to killing by chlorambucil \((p < 0.01, \text{Fig. 5C})\) or cisplatin \((p < 0.01, \text{Fig. 5D})\), although in each case, the double mutants already displayed noticeable sensitivity.

The Enhanced Sensitivity Is at the Cell Permeability Level

If the enhanced sensitivity of the \(RNR3\)-lacZ assay by inactivation of \(PDR\) and \(CWP\) genes is indeed due to increased
cell permeability instead of some other unknown factors, one would argue that pdr and cwp mutations should not affect the RNR3-lacZ induction by DNA damage that does not involve cell permeability. To test this prediction, we treated wild-type and various mutant cells with UV irradiation at a dose known to induce RNR3-lacZ. As shown in Figure 6, inactivation of PDR (pdr5 snq2), CWP (cwp1 cwp2), or both pathways had no obvious effect on the RNR3-lacZ expression.

Specificity of Enhanced RNR3-lacZ Sensitivity to Toxic Compounds by Cell Permeability Mutations

Since we have previously shown that the RNR3-lacZ assay only detects genotoxicants and does not respond to toxic compounds that kill yeast cells by means other than damaging DNA (Jia et al., 2002), one would predict that the enhanced cell permeability by cwp and pdr mutations should not affect the RNR3-lacZ response to those nongenotoxic chemicals. We tested three such well-characterized antibiotics, namely L-canavanine, tetracycline, and kanamycin. None of the three antibiotics induced RNR3-lacZ to a noticeable level, and most importantly, the cwp pdr double and the quadruple mutants did not display enhanced RNR3-lacZ activity after treatment with these antibiotics at any concentrations examined (Figs. 7A–C). Interestingly, the cell permeability mutations variably increased the cellular sensitivity to killing by L-canavanine (Fig. 7D) and tetracycline (Fig. 7E), but their effects on kanamycin-induced killing is not obvious (Fig. 7F). For example, at the highest L-canavanine dose tested, each double mutant showed ~50% survival compared with 90% survival of wild-type cells, whereas the survival rate in the quadruple mutant was only 30% (Fig. 7D), suggesting an additive effect of the two separate cell permeability pathway mutations on the bioavailability of intracellular tested agents.
Comparison of the Detection Limit between Yeast RNR3-lacZ Assay and Ames Test

The pdr5 snq2 cwp1 cwp2 quadruple mutation significantly improved the RNR3-lacZ sensitivity to detecting genotoxic compounds examined. Quantitative analysis indicates that the combined enhancement, as judged by the minimal dose of testing agents capable of two fold induction in the RNR3-lacZ assay, achieved 1.6-fold for MMS, 14-fold for 4-NQO, 24-fold for phleomycin, 11-fold for chlorambucil, and 3.4-fold for cisplatin (Table 1). The improvement of yeast cell permeability alone makes the RNR3-lacZ assay ninefold more sensitive to MMS and chlorambucil and fivefold more sensitive to 4-NQO than the Ames test (Table 1). There are no Ames test data available on phleomycin in the database. We performed Ames test with both TA98 and TA100 strains and found that phleomycin doses up to 33 μg/plate (a lethal dose) did not result in apparent increase in mutagenesis and that addition of the S9 liver extract did not make a difference (data not shown).

**DISCUSSION**

The current study investigated the effect of the PDR pathway on the sensitivity of the RNR3-lacZ testing system to detect genotoxicants in S. cerevisiae. PDR is known to affect cellular transport and sensitivity to various toxic chemicals. Since genes involved in PDR pathways can be modified genetically to alter cellular permeability, we hypothesized that inactivation
of certain PDR genes would be able to enhance the RNR3-lacZ sensitivity to selected genotoxic agents, which is indeed the case. Furthermore, several conclusions can be drawn from this study. First, individual deletion of major ABC transporter genes had minimal effect on cell survival and RNR3-lacZ induction with the exception of the effect of snq2 mutation on DNA damage induced by 4-NQO. Second, simultaneous deletion of two PDR genes could have an additive effect, even though deletion of either gene may not cause an enhanced sensitivity. This observation indicates overlapping functions of PDR genes in the protection of cells against drug entry. Third, it appears that the increased RNR3-lacZ activity can be detected at doses lower than those that would induce an obvious killing effect, suggesting that the RNR3-lacZ assay often is more sensitive than the cell survival–based assays. Finally, the enhanced sensitivity is deemed to be due to cell permeability since deletion of PDR genes does not affect RNR3-lacZ expression induced by UV irradiation, which does not require cell permeability.

To our surprise, deletion of the two PDR transcriptional regulators PDR1 and PDR3, known to be required for the activation of a large number of PDR genes, has different effects on the RNR3-lacZ assay, while the deletion of PDR3 compromises RNR3-lacZ induction by all three tested chemicals, deletion of PDR1 has no effect on the RNR3-lacZ assay, regardless of wild type or pdr3 background. The fact that deletion of PDR3 affects the induction of different DNA damage–inducible genes including RNR3, MAG1, and DDI1 suggests that it is involved in an SOS-like transcriptional regulation (Fu et al., 2008). Nevertheless, since the pdr1 pdr3 double mutant displayed an increased sensitivity to killing by 4-NQO and phleomycin, it is conceivable that Pdr3 is required for the transcriptional regulation of RNR3 in response to DNA damage and that this effect is specific to transcription-based assays, such as RNR3-lacZ. Hence, results obtained from killing experiments suggest that the pdr1 pdr3 double mutant has increased intracellular bioavailability of tested toxic agents and hence remains an attractive strain to explore other toxicity-based assays.

Perhaps the most exciting observation in this study is the synergistic enhancement of the RNR3-lacZ assay by simultaneous inactivation of both PDR and CWP genes. This investigation is based on the hypothesis that cellular protections provided by efflux pumps and CWPs are two distinct mechanisms. Confirmation of the above hypothesis through this study is not only of great significance in theory but also offers an excellent hyperpermeable yeast strain to enhance the detection of genotoxic chemicals when existing in low abundance. Furthermore, the overlapping functions of PDR and CWP pathways in preventing entry of genotoxic compounds into cells is best illustrated by our observation that while the inactivation of either pathway resulted in barely noticeable enhancement of the RNR3-lacZ activity by chlorambucil, simultaneous inactivation of both pathways caused a 28-fold increase in the RNR3-lacZ induction by the same compound.

Recently, several yeast transcription–based reporter systems have been developed (Afanassiev et al., 2000; Benton et al., 2007; Jia et al., 2002) and the recent focus was to improve the sensitivity of these assays through genetic modifications of the host cells, including inactivation of DNA repair pathways (Benton et al., 2008; Jia and Xiao, 2003), metabolic activation, and compound solubility (Walsh et al., 2005). These manipulations are expected to apply to narrowly selected compounds. In contrast, improvement of cell permeability is anticipated to have a broad range of applications. Of great significance is the fact that multidrug resistance (MDR) genes in mammalian cells also encode membrane transporters (Gros et al., 1986) homologous to the yeast PDR genes (Raymond et al., 1992), making this study potentially applicable to public health. It is rather exciting to notice that genetic modifications of the yeast cell permeability alone make the RNR3-lacZ assay more sensitive than Ames test in detecting most genotoxic agents included in this study. An extreme case is phleomycin in which inactivation of both CWP and PDR pathways results in a stunning 24-fold enhancement of the RNR3-lacZ assay sensitivity so that it only requires less than 2 μg/ml phleomycin to induce more than twofold RNR3-lacZ activity. In contrast, phleomycin is unable to induce mutagenesis in the Ames test under all experimental conditions despite its strong cytotoxic effect on the host cell. However, we noticed a report (Hall, 1985) in which 5 μg/plate phleomycin was able to cause an up to twofold increase in an Escherichia coli Trp+ reversion assay in the presence of pKM101.

Our observation that yeast cell permeability mutations do not enhance the RNR3-lacZ activity in response to nongenotoxic...
compounds is consistent with the predicted \textit{RNR3-lacZ} specificity. Meanwhile, the cell permeability mutations do enhance cytotoxicity of these nongenotoxic agents, suggesting that the hyperpermeable quadruple mutant created in this study is applicable to detect not only genotoxic agents but also nongenotoxic chemicals.

**SUPPLEMENTARY DATA**

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

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YEAST MUTANT WITH ENHANCED PERMEABILITY


