

# Enhancing Drug Accumulation in *Saccharomyces cerevisiae* by Repression of Pleiotropic Drug Resistance Genes with Chimeric Transcription Repressors

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## ABSTRACT

Yeast is a powerful model system for studying the action of small-molecule therapeutics. An important limitation has been low efficacy of many small molecules in yeast due to limited intracellular accumulation. We used the DNA binding domain of the pleiotropic drug resistance regulator pleiotropic drug resistance 1 (Pdr1) fused in-frame to transcription repressors to repress Pdr1-regulated genes. Expression of these chimeric regulators conferred dominant enhancement of sensitivity to a different class of compounds and led to greatly diminished

levels of Pdr1p-regulated transcripts, including the yeast *p*-glycoprotein homolog Pdr5. Enhanced sensitivity was seen for a wide range of small molecules. Biochemical measurements demonstrated enhanced accumulation of rhodamine in yeast cells expressing the chimeric repressors. These repressors of Pdr1p-regulated transcripts can be introduced into large collections of strains such as the *Saccharomyces cerevisiae* deletion set and enhance the utility of yeast for studying drug action and for mechanism-based drug discovery.

Yeast cells are frequently insensitive to small molecules that target essential proteins. The lack of response is often not due to insensitivity of the yeast target to an inhibitor. Rather, yeast cells fail to accumulate small molecules to biologically effective concentrations. Although the presence of the yeast cell wall may be an impediment in some cases, yeast cells are also very effective at reducing intracellular concentration of toxic small molecules using a large number of proteins homologous to transport proteins (Moye-Rowley, 2003; Ernst et al., 2005). The expression of a diverse array of proteins that can transport toxic molecules is probably an important property for the survival of yeast cells in the wild, but it creates difficulties in adapting yeast as a model for studying the action of potential therapeutic agents.

Studies of yeast cells selected for high levels of resistance to otherwise toxic molecules led to the discovery of regulatory networks that control the expression of many drug efflux proteins. Because some of these mutants were resistant to multiple classes of agents, the altered genes were termed

pleiotropic drug resistance (Pdr) genes (Balzi and Goffeau, 1991). Subsequent analyses showed that the mutations conferring pleiotropic drug resistance were often within genes that regulated the expression of drug transport proteins (Balzi et al., 1987; Katzmann et al., 1994; Mahé et al., 1996). Two major regulators, Pdr1p and Pdr3p, coordinately regulate a set of proteins, including several ABC transporters that are homologous to mammalian transporters such as *ABCB1* (MDR1) (Moye-Rowley, 2003). These ABC transporters were shown to confer drug resistance when overexpressed and, in some cases, drug hypersensitivity when the structural genes encoding the transporters were deleted. Because yeast cells express a wide range of transporters that have overlapping substrate preferences, drug hypersensitivity in mutants deleted for drug transporters was frequently limited to a narrow range of substrates. One important exception is mutants carrying deletions of *PDR5* (Balzi et al., 1994; Leonard et al., 1994). Strains lacking *PDR5* show hypersensitivity to a variety of small molecules including cycloheximide, herbicides, and other enzyme inhibitors (Golin et al., 2003; Mitterbauer et al., 2003). However, sensitivity to many other small molecules is unaffected by deletion of *PDR5* (see Fig. 8 for examples).

Despite problems relating to drug accumulation, *Saccha-*

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**ABBREVIATIONS:** Pdr, pleiotropic drug resistance; SD-leu, synthetic dextrose lacking leucine; PCR, polymerase chain reaction; DBD, DNA binding domain; ORF, open reading frame; GFP, green fluorescent protein; bp, base pair(s); FDR, false-discovery rate; PDRE, pleiotropic drug resistance response element; TPI, triose phosphate isomerase promoter.

*Saccharomyces cerevisiae* strains have been successfully used to analyze mechanisms of cytotoxicity for a variety of anticancer drugs (Nitiss and Heitman, 2007). The ability to analyze drug action in yeast frequently depends on the introduction of mutations enhancing drug accumulation. One strategy for overcoming this problem is to introduce multiple mutations as was done for a large screening project carried out by the National Cancer Institute. They used yeast strains harboring simultaneous deletions of *PDR1*, *PDR3*, and *ERG6* to identify small molecules that specifically affected yeast cells carrying mutations in DNA repair genes (Dunstan et al., 2002).

An incentive for overcoming the insensitivity of yeast cells to small molecules is the development of a set of yeast-based tools applicable to studying drug action (Winzeler et al., 1999). Identifying the reduced fitness of strains grown in the presence of a drug enables efficient genome-wide screen for drug targets, other pathways targeted by a drug, rate-limiting components of targeted pathways, and the identification of alternate or parallel biochemical pathways (Giaever et al., 1999).

In this article, we describe chimeric transcriptional regulators using the DNA binding domain of Pdr1 fused to the transcriptional corepressors Sin3 (Wang and Stillman, 1993; Kadosh and Struhl, 1998) or Cyc8 (Zhang and Reese, 2004; García-Sánchez et al., 2005). Expression of these novel regulators renders yeast cells sensitive to a variety of small molecules. We demonstrated that expression of these fusions effectively reduces the expression of transporters regulated by Pdr1/Pdr3. We show that enhanced sensitivity to small molecules is probably due to changes in the expression of transporters and directly demonstrate that pdr1DBD-repressor fusions increase intracellular accumulation of a small molecule. Our results show that chimeric transcription factors represent a novel effective strategy to circumvent the natural multidrug resistance found in yeast cells and can be used to greatly enhance the effectiveness of yeast as a system for studying drug action.

## Materials and Methods

**Strains and Media.** The *S. cerevisiae* strain BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) and KANMX4 ORF deletion derivatives were purchased from Open Biosystems (Huntsville, AL). Standard yeast lithium acetate transformation techniques were used (Gietz et al., 1992). Strains transformed with the fusion constructs were selected for on synthetic dropout synthetic dextrose lacking leucine (SD-leu) media (Nitiss et al., 1992). Drug sensitivity was performed using SD media with appropriate nutrients deleted as required for the selection of plasmids. Experiments with Gal1 overexpression also used synthetic media with 2% galactose replacing dextrose. The strain JN362a is moderately hypersensitive to a variety of inhibitors, including etoposide and 4'-(9-acridinylamino)methanesulfon-*m*-anisidide (amsacrine) (Nitiss and Wang, 1988).

**Vector Construction.** For cloning purposes, PCR primers were designed with appropriate restriction enzyme sites at the ends or with homology to adjacent sequences for second-round PCR reactions (primer sequences available upon request). Yeast genomic DNA (Promega, Madison, WI) was used as the PCR template for cloning and for generating Northern blot probes.

A segment of the *PDR1* gene including DNA binding domain (DBD) of yeast *PDR1* (nucleotides 1–621) was amplified using the Expand High Fidelity PCR kit (Roche, Indianapolis, IN) and subcloned into the BamHI/EcoRI sites of pBluescript II SK (Stratagene, La Jolla, CA). The entire *SIN3* ORF was PCR-amplified and cloned

in-frame downstream of the *PDR1*-DBD using EcoRI and XhoI sites of pBluescript. The entire ORF of *CYC8* was cloned downstream of the *PDR1*-DBD using the same scheme. The *PDR1*-GFP fusion was constructed by PCR amplification of GFP from the plasmid pIRES-EGFP (Clontech, Mountain View, CA) using primers homologous to the *PDR1*-DBD. *PDR1*-DBD amplification used primers containing homology to GFP. The products were mixed, annealed, and followed by a second round of amplification using *PDR1* and GFP-specific primers. The product was cloned into pBluescript using BamHI and XhoI. The *PDR1*-DBD-Sin3, -Cyc8, and -GFP fusions were excised using BamHI/XhoI and cloned into pYX142, a yeast centromeric plasmid, under the control of the triose phosphate isomerase (*TPI1*) promoter and containing the *LEU2*-selectable marker. The vector-overexpressing *PDR5* was constructed using the plasmid pYES2 and a PCR product containing the entire *PDR5* ORF. Where appropriate, pYX vectors with different selectable markers were used as empty vector control plasmids.

**Integrating Fusion Construct.** The polyA signal and *LEU2* gene were amplified from pYX142 and cloned into the XhoI site downstream of the fusion constructs in pBluescript (SalI/XhoI). A 455-bp fragment containing the 3' XhoI site within the *PDR1* ORF was amplified using primers that generated SalI sites at the ends of the PCR product and cloned into the XhoI site downstream of the *LEU2* marker. A 390-bp fragment 160 bp upstream of the *PDR1* start codon (containing a natural XhoI site) was amplified using primers that were homologous to *TPI1* promoter at the primer 3' end. The product was annealed to another PCR product containing the *TPI1* promoter and a 3' BamHI site. A second round of PCR using external primers produced a 5'pdr1Δ fragment (upstream of *PDR1*) fused to the *TPI1* promoter. This BglII/BamHI piece was cloned into the BamHI site upstream of the fusion constructs in pBluescript. XhoI digestion releases a DNA fragment of approximately 300 bp of homology both 5' and 3' of the *PDR1* gene, the *TPI1* promoter, the pdr1:Cyc8 fusion construct, and the *LEU2*-selectable marker. This fragment was used for transformation and homologous integration in yeast. Correct integration at the *PDR1* locus was verified by PCR.

**Drug Sensitivity.** For growth on media containing drug, logarithmically growing cells were diluted to  $OD_{600} = 0.3$ , and 10-fold serial dilutions were spotted onto synthetic media containing the indicated drug. For all compounds added to media containing agar, drug was added when the media was at approximately 50°C, followed by immediate pouring of the agar into plastic plates. Plates were incubated at 30°C for 2 to 3 days and photographed. Drugs examined in this way included cycloheximide (Sigma, St. Louis, MO), camptothecin (A.G. Scientific, San Diego, CA), etoposide (Bedford Laboratories, Bedford, OH), miconazole (Sigma), and Rhodamine 6G (Invitrogen, Carlsbad, CA). An additional set of compounds termed "cancer plate" containing 80 clinical and experimental anticancer drugs was obtained from Discovery Microsource (available at <http://www.msdiscovery.com/>). Compounds on the plate were obtained as 10 mM stock solutions in dimethyl sulfoxide. Drug sensitivity for these compounds (Fig. 8) was assessed using a different protocol than the protocol described above. An overnight culture of the appropriate yeast strains was diluted to  $2 \times 10^6$  cells/ml. A portion of the culture (200  $\mu$ l) was dispensed into 96-well plates. Drug or dimethyl sulfoxide was added, and the 96-well plate was incubated without shaking for 24 h at 30°C. After the incubation, 10-fold serial dilutions of the final cultures were spotted on drug-free SD-leu media and grown for 48 h at 30°C. Clonogenic survival assays with etoposide were carried out as described previously (Nitiss et al., 1992).

**Analysis of GFP Localization.** GFP localization and 4,6-diamidino-2-phenylindole staining of yeast nuclei was performed as described by Huh et al. (2003) using the *PDR1*-GFP fusion described above.

**Rhodamine-6-G Uptake Assay.** Rhodamine uptake assays were performed using a modified procedure of van den Hazel et al. (1999). Approximately  $2.8 \times 10^8$  logarithmically growing cells were washed

three times and resuspended in 2 ml of buffer A (50 mM HEPES/NaOH, pH 7.0). Aliquots (200  $\mu$ l) were taken for measuring background cell fluorescence. Rhodamine-6-G was added to a final concentration of 5 mM, and 200- $\mu$ l aliquots were taken every 10 min for 1 h. For each time point, cells were washed three times with ice-cold buffer A, and Rhodamine-6-G fluorescence was then measured using a CytoFluor 2300 (Applied Biosystems, Foster City, CA) with excitation filter at 530 nm and emission filter at 590 nm.

**Affymetrix GeneChip Analysis.** Total RNA was extracted from logarithmic yeast cultures using the RiboPure-Yeast protocol (Ambion, Austin, TX) according to the manufacturer's instructions. RNA quality was confirmed by UV spectrophotometry and by analysis on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Ten micrograms of total RNA was processed in the St. Jude microarray core facility according to the Affymetrix (Santa Clara, CA) eukaryote target labeling protocol revision 4 (available at [http://www.affymetrix.com/support/technical/manual/expression\\_manual.affx](http://www.affymetrix.com/support/technical/manual/expression_manual.affx)). Labeled targets were hybridized to Affymetrix YG\_S98 GeneChip arrays, which interrogate  $\sim$ 7000 *S. cerevisiae* ORFs and transcripts. Signal values and detection calls were determined using the default parameters in the Affymetrix GCOS software (version 1.4). Signals were scaled to a 2% trimmed mean of 500. Probeset annotations (March, 2007) were obtained from the Affymetrix website (available at <http://www.affymetrix.com/analysis/index.affx>). All microarray data have been submitted to Gene Expression Omnibus (available at <http://www.ncbi.nlm.nih.gov/geo/>) (GSE8326).

Three replicate cultures of each yeast strain were used to identify differentially expressed transcripts. Signal values were log<sub>2</sub>-transformed before analysis. The local pooled error *t* test (Jain et al., 2003) was used to compare transcript levels in cultures containing Pdr1-fusion constructs with those containing an empty vector (pYX142). To adjust for multiple hypothesis testing, the method of Benjamini and Hochberg (1995) was used to estimate the false-discovery rate (FDR). Transcripts with differential expression were defined as those with a minimum of 2-fold difference in magnitude and with an FDR < 0.05. Statistical analyses were performed using the Array-Analyzer module in S-PLUS 6.2 (Insightful, Seattle, WA).

**Northern Analysis.** Total RNA was isolated using the same procedure used for isolating RNA for microarray analysis. Electrophoresis of RNA, transfer to nylon membranes, and hybridization was performed using standard techniques (Sambrook et al., 1989). Probes specific for *PDR5*, *YOR1*, and yeast actin (loading control) were purified by gel electrophoresis before labeling by random priming.

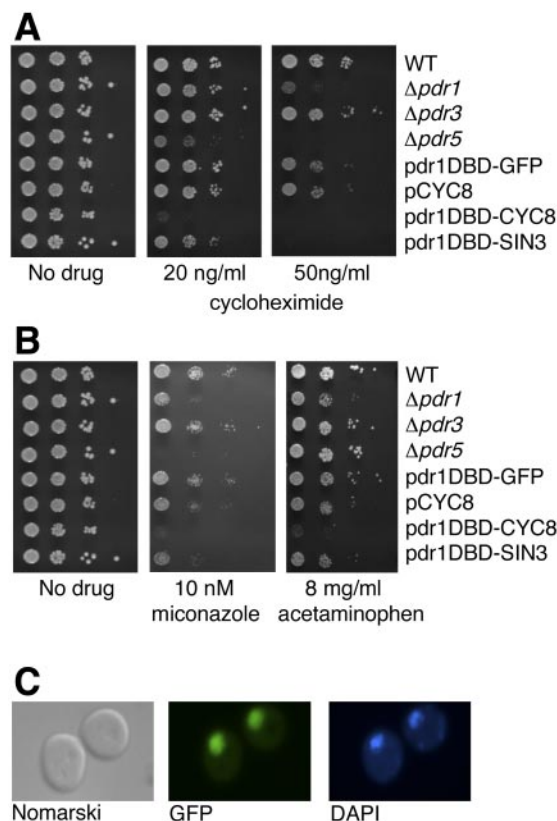
## Results

The redundancy of transporters conferring pleiotropic drug insensitivity in yeast implies that repression or inhibition of several drug efflux proteins is required to enhance intracellular accumulation of small molecules. Two major regulators of the Pdr network, Pdr1p and Pdr3p, are DNA binding transcriptional activators that recognize the same DNA sequence (TTCGGCGGAA, termed a PDR response element, PDRE) (Katzmann et al., 1994, 1996). A dominant-negative allele of these activators might represent an effective strategy for blocking the expression of proteins regulated by this network. We hypothesized that we could deliver a potent repressor of transcription to promoters regulated by Pdr1p and Pdr3p by fusing the DNA binding domain of Pdr1p (or Pdr3p) to potent transcriptional repressors. We chose two yeast transcriptional repressors, Cyc8p (Keleher et al., 1992) and Sin3p (Wang and Stillman, 1993). Cyc8p and Sin3p are components of distinct histone deacetylase complexes (Hda1p and Rpd3p, respectively) (Heinzel et al., 1997; Davie et al., 2003), and neither protein interacts with DNA except by

interacting with other sequence-specific DNA binding proteins (Kasten et al., 1997).

Details of the construction of the chimeric transcriptional repressors are described under *Materials and Methods*. In brief, both chimeric repressors were expressed from the constitutive *TPI1* promoter carried on the yeast single-copy vector pYX142. The first 621 nucleotides of the *PDR1* coding sequence was fused with either the entire *SIN3* coding sequence (yielding plasmid pdr1DBD-SIN3) or the entire *CYC8* coding sequence (yielding plasmid pdr1DBD-CYC8). A control construct carried the first 621 nucleotides of the *PDR1* coding sequence fused to GFP to assess the importance of the repressor for efficient blocking of Pdr gene expression. We also expressed the coding sequence of *CYC8* with the *TPI1* promoter to assess the effect of *CYC8* overexpression (not conjugated to a DNA binding domain) on drug sensitivity.

We first tested the effects of the chimeric repressors on sensitivity to the translation inhibitor cycloheximide, a compound that is frequently used to study the Pdr network in yeast (Balzi et al., 1987; Meyers et al., 1992) (Fig. 1A). Wild-type cells (BY4741) carrying either pdr1DBD-SIN3 or pdr1DBD-CYC8 were significantly more sensitive to cyclo-



**Fig. 1.** Dominant interference with pleiotropic drug resistance using Pdr1-repressor fusions. A, cycloheximide sensitivity of yeast carrying the pdr1DBD-repressor fusion constructs compared with yeast strains with ORF deletions of various *PDR* genes. Logarithmically growing yeast cultures were serially diluted and spotted onto plates containing 20 or 50 ng/ml cycloheximide. Plates were incubated at 30°C for 2 days and then photographed. B, sensitivity of yeast carrying pdr1DBD-repressor fusions to other drugs was determined by spotting diluted cell cultures onto agar plates containing miconazole and acetaminophen at the concentrations indicated. The drug-sensitivity experiments were carried out at the same time as those shown in A, and the same no-drug data are displayed. C, Pdr1DBD-GFP fusion was detected in the nucleus by fluorescent microscopy. The nucleus was also stained with 4,6-diamidino-2-phenylindole.



heximide than cells with an empty vector. Cells carrying pdr1DBD-CYC8 were unable to grow in medium containing 20 ng/ml cycloheximide, whereas cells carrying pdr1DBD-SIN3 were somewhat less sensitive but failed to grow in media containing 50 ng/ml cycloheximide. By comparison,  $\Delta pdr1$  cells showed reduced growth only at 50 ng/ml cycloheximide, whereas  $\Delta pdr5$  cells exhibited reduced growth at 20 ng/ml cycloheximide. It is noteworthy that cells carrying pdr1DBD-GFP or cells overexpressing *CYC8* showed only modest reductions in growth at 50 ng/ml cycloheximide. One reason that the pdr1DBD-GFP fusion might fail to repress genes of the Pdr network is improper localization. To rule out this possibility, we showed that pdr1DBD-GFP localized to the nucleus (Fig. 1C). These results indicate that the Pdr1DBD-repressor fusions can have an impact on sensitivity to cycloheximide and that both the Pdr1 DNA binding domain and the presence of a repressor are required for enhanced cycloheximide sensitivity.

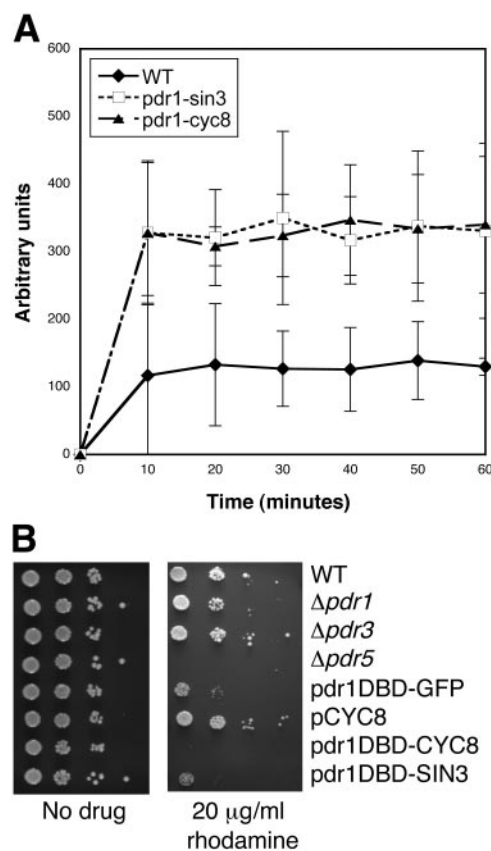
To demonstrate that the achieved drug sensitivity is not limited to a specific compound or biochemical pathway, we tested the same strains for sensitivity to several unrelated drugs targeting different biological processes, including inhibitors of ergosterol synthesis such as miconazole and the analgesic acetaminophen, which has been shown to inhibit the growth of yeast cells at high concentrations (Srikanth et al., 2005). Cells expressing pdr1DBD-CYC8 showed sensitivity to miconazole equivalent to that seen in  $\Delta pdr5$  cells, and pdr1DBD-SIN3-bearing cells were strongly inhibited by 10 nM miconazole. The other strains tested exhibited less sensitivity at this drug concentration (Fig. 1b). Likewise, pdr1DBD-CYC8 strains failed to grow in media containing 8 mg/ml acetaminophen and were the most sensitive strains tested. As observed with cycloheximide, strains carrying pdr1DBD-GFP or cells overexpressing *Cyc8p* without a Pdr1 DNA binding domain showed wild-type levels of sensitivity to these two agents. Cells carrying pdr1DBD-CYC8 were also hypersensitive to other compounds (Fig. 8).

To determine whether increased drug sensitivity depends on higher intracellular concentrations of inhibitors, we directly measured intracellular accumulation of rhodamine-6G (van den Hazel et al., 1999) in yeast cells expressing pdr1DBD-SIN3 or pdr1DBD-CYC8 compared with cells carrying a pYX empty vector. Cells carrying either fusion accumulated approximately 3-fold higher levels of rhodamine-6G compared with cells carrying a pYX empty vector (Fig. 2A). Exposure to 20  $\mu$ g/ml rhodamine-6G completely inhibited the growth of cells carrying pdr1DBD-CYC8 and greatly inhibited the growth of cells carrying pdr1DBD-SIN3, whereas the growth of wild-type cells was affected to a much lesser extent (Fig. 2B), indicating an enhanced biological effect of rhodamine-6G on repressor fusion-bearing cells. This result directly demonstrates that the pdr1DBD-repressor fusions can result in alterations in yeast cell accumulation of a small molecule.

We predicted that expression of pdr1DBD-repressor fusions should enhance drug accumulation by down-regulating the expression of Pdr1-regulated genes. Furthermore, because the experiments presented above were carried out in strains carrying a wild-type *PDR1* gene, we predicted that the down-regulation of Pdr1-regulated genes could overcome transcriptional activation by wild-type Pdr1. Because genes regulated by Pdr1 and Pdr3 show different dependence on

Pdr1 or Pdr3 loss-of-function (Moye-Rowley, 2003) we were interested in determining the transcriptional targets of the Pdr1DBD-repressor fusions.

We carried out microarray analysis using Affymetrix YG\_S98 GeneChip arrays and assessed whether the Pdr1DBD-repressor fusions altered the expression of genes regulated by Pdr1. For this analysis, we compared the effect of the Pdr1DBD-repressor fusions with genes up-regulated by the expression of a dominant allele, *PDR1-3*, that confers a hyper-resistant phenotype to cycloheximide and other agents and results in the overexpression of Pdr1 target genes (Moye-Rowley, 2003; Jungwirth and Kuchler, 2006). As tabulated in Fig. 3, three genes up-regulated by *PDR1-3* were significantly down-regulated by both Pdr1DBD-repressor fusions: *ICT1*, a protein of unknown function whose deletion enhances sensitivity to Calcofluor white; *PGA3*, an essential protein that localizes to the endoplasmic reticulum; and the drug transporter *PDR5*. One gene, *HXX1* (hexokinase) that was up-regulated by *PDR1-3* was also up-regulated by both promoter fusions. Up-regulation of YGR243W was also seen, although the effect was not significant at an FDR < 0.05. Several other genes showed reduced transcription in one but not both repressor fusions, whereas another



**Fig. 2.** Enhanced intracellular concentration of rhodamine 6G in pdr1-repressor fusion strains. A, cell-associated fluorescence of rhodamine 6G. Cells from logarithmically growing yeast cultures carrying either a vector control or plasmids expressing pdr1DBD-repressor fusions were assayed for the ability to accumulate rhodamine-6G. Cell-associated fluorescence due to rhodamine-6G was measured by spectrofluorometry. Error bars indicate  $\pm$ S.D. of three independent experiments. B, sensitivity of yeast cultures carrying pdr1DBD-repressor fusions or deletions of Pdr genes was assayed by spotting diluted cell cultures onto plates containing rhodamine 6G. The drug-sensitivity experiments were carried out at the same time as those shown in Fig. 1A, and the same no-drug data are displayed.

set of genes showed reduced expression that was not significant at a FDR < 0.05. Also shown in Fig. 3 is the analysis of the effect of Pdr1DBD-CYC8 in a strain carrying a deletion of the *PDR1* gene. In this case, six additional genes up-regulated by *PDR1-3* were down-regulated by the combination of expression of Pdr1DBD-CYC8 and deletion of *PDR1*. It is interesting that not all of the genes identified by DeRisi et al. (2000) that were up-regulated by *PDR1-3* contained a PDRE; however, all genes that were down-regulated by the combination of expression of Pdr1DBD-CYC8 and deletion of *PDR1* contained a PDRE. The genes that were up-regulated also lack a PDRE. Taken together, these results suggested that at least some Pdr1 target genes were down-regulated by the Pdr1DBD-repressor fusions and that deletion of *PDR1* enhanced the effectiveness of the Pdr1DBD-repressor fusions. The full set of expression data are available online (<http://www.ncbi.nlm.nih.gov/geo/>) (GSE8326).

We confirmed some of the results obtained with the microarray studies by Northern analysis using probes for two target genes, *PDR5* and *YOR1*. Both genes are well-established *PDR1* targets. The microarray analysis showed that *PDR5* expression was reduced by both Pdr1DBD:repressor fusions, whereas *YOR1* was only significantly reduced by Pdr1DBD-SIN3. However, by Northern analysis, Pdr1DBD-CYC8 clearly reduced the expression of both *PDR5* and *YOR1* (Fig. 4). Taken together, these results indicate that multiple

*PDR1* targets are down-regulated by Pdr1DBD:repressor fusions. The Northern analysis also demonstrated efficient down-regulation in the strain carrying both Pdr1DBD-CYC8 and  $\Delta PDR1$ . Given the low level of expression of these two target genes in the Pdr1DBD-CYC8 strain, it was not possible to determine whether deletion of *PDR1* enhanced the silencing of these two target genes.

Although the Pdr1DBD:repressor fusions are clearly dominant, deletion of *PDR1* might confer an additional advantage in conferring drug sensitivity when coupled with Pdr1DBD:repressor fusions. To assess this possibility, we examined the sensitivity of Pdr1DBD:repressor fusion bearing strains to etoposide, a Top2-targeting drug. Etoposide does not readily enter yeast cells, and our previous results indicate that single mutations such as  $\Delta erg6$  or  $\Delta pdr5$  failed to result in etoposide sensitivity. Figure 5A shows that strains carrying a pYX empty vector,  $\Delta pdr1$  or  $\Delta pdr5$ , are not sensitive to etoposide. Deletion of *PDR1* combined with expression of Pdr1DBD-CYC8 completely blocks growth on 100  $\mu$ g/ml etoposide, whereas deletion of *PDR5* along with Pdr1DBD-CYC8 exerts a lesser effect. This result clearly shows that deletion of *PDR1* greatly enhances the effectiveness of the Pdr1DBD:repressor fusions.

We further confirmed the etoposide sensitivity by carrying out clonogenic survival assays with various BY4741 deriva-

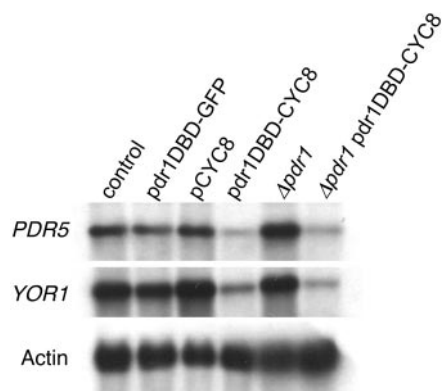
Gene Symbol	Gene Description	Systematic ID	Pdr1-Cyc8 $\Delta pdr1$	Pdr1-Cyc8	Pdr1-Sin3
HXK1	Hexokinase	YFR053C	5.0	3.7	6.2
ICT1	Function unknown	YLR099C	-5.8	-3.0	-2.7
PDR5	ABC transporter	YOR153W	-5.3	-4.8	-4.8
GRE2	NADPH-dependent methylglyoxal reductase	YOL151W	-8.4	-3.2	-1.7
YOR1	ABC transporter	YGR281W	-8.5	-1.6	-2.4
PDR16	Phosphatidylinositol transfer protein	YNL231C	-5.6	-2.6	-2.0
YGP1	Cell wall-related secretory glycoprotein	YNL160W	-10.9	-2.1	-1.4
SNQ2	ABC transporter	YDR011W	-4.4	-1.3	-2.0
---	Putative aryl alcohol dehydrogenase	YPL088W	-2.1	-2.3	-1.3
---	Putative protein of unknown function, potential Cdc28p substrate	YGR035C	-6.2	-1.8	1.2
---	Putative protein of unknown function found in mitochondria	YLR346C	-2.0	-2.0	-1.2
MET17	O-acetyl homoserine-O-acetyl serine sulphydrylase	YLR303W	-2.0	-2.0	-1.3
COS10	Protein of unknown function, member of the DUP380 subfamily	YNR075W	-1.9	1.2	-1.3
DSE4	Secreted protein similar to glucanases	YNR067C	-1.4	1.3	-1.7
PDR15	ABC transporter	YDR406W	-1.4	1.4	1.2
REV1	Deoxycytidyl transferase	YOR346W	-1.2	-1.3	-1.4
SCW11	Cell wall protein with similarity to glucanases	YGL028C	-1.2	1.0	-1.1
---	Putative protein of unknown function	YLL056C	-1.1	-1.1	-1.7
---	Putative protein of unknown function	YLR413W	1.1	-1.6	-1.8
---	Putative protein of unknown function	YOR152C	1.1	1.1	-1.1
FRE4	Ferric reductase	YNR060W	1.2	-1.2	-1.2
RTA1	Protein involved in 7-amincholesterol resistance	YGR213C	1.2	-2.6	-1.4
SLI1	N-acetyltransferase	YGR212W	1.3	-1.4	-1.2
---	Putative protein of unknown function	YGR243W	1.4	5.1	4.4
RSB1	Putative integral membrane transporter or flippase	YOR049C	2.2	-1.7	2.2

**Fig. 3.** Expression analysis in cells containing pdr1DBD-fusion constructs. Gene expression profiles were obtained by hybridization to Affymetrix YG\_S98 GeneChip arrays. Profiles were obtained from three independent RNA isolations for each strain tested. Data presented are the expression profiles of genes that are up-regulated by the *pdr1-3* allele. The last three columns show the mean fold change relative to that of empty vector in yeast strains expressing PDR1-fusion repressors. -, expression was decreased relative to control. Ratios highlighted in yellow were significant at an FDR < 0.05. Genes highlighted in blue contain PDR response elements in their promoter regions as reported previously (DeRisi et al., 2000; Hikkel et al., 2003). Three genes, (*COS10*, *PDR5*, and *YOR1*) had multiple probe sets on the YG-S98 array. For these genes, we reported the median change relative to vector and highlighted that ratio when at least one of the probe sets achieved the FDR < 0.05. In addition to the conditions reported here, we also analyzed the effect of deletion of *PDR1* in cells carrying an empty vector. The full set of expression data are available online (at <http://www.ncbi.nlm.nih.gov/geo/>) (GSE8326).

tives. Wild-type BY4741 is insensitive to etoposide, as are  $\Delta erg6$  derivatives. Expression of Pdr1DBD-CYC8 from a plasmid (with a wild-type chromosomal copy of *PDR1*) is also etoposide-insensitive, consistent with the spot tests shown in Fig. 5A. The integrated Pdr1DBD-CYC8 construct that deletes the chromosomal copy of *PDR1* is very sensitive to etoposide, with concentrations greater than 100  $\mu\text{g/ml}$  reducing viability below the viable titer at the time of drug addition. Strain JN362a is etoposide-sensitive when it also carries a mutation compromising DNA repair pathways but is insensitive when it is repair-proficient. The integrated Pdr1DBD-CYC8 strain is the first well-defined *S. cerevisiae* strain we have tested that can be killed by etoposide even in the absence of any mutations conferring DNA repair defects.

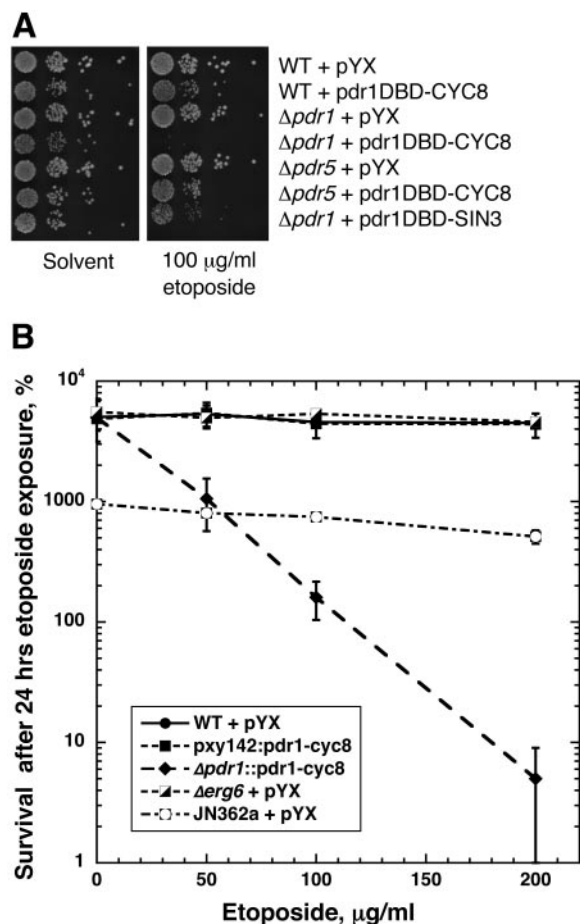
Because the *PDR1* gene seems to regulate the expression of genes that may affect membrane trafficking and drug transporters, the effectiveness of the Pdr1DBD:repressor fusions may affect the disposition of drug transport proteins and other membrane proteins. To test this possibility, we constructed a vector that expressed the *PDR5* transporter from the *GAL1* promoter. If mislocalization of membrane proteins is an important effect of the Pdr1DBD:repressor fusions, expression of *PDR5* from a different promoter would be insufficient to restore wild-type drug resistance. However, we found that cells that express *PDR5* from the *GAL1* promoter become cycloheximide-resistant even when Pdr1DBD-CYC8 or Pdr1DBD-SIN3 is expressed (Fig. 6). This result suggests that the major effect of the repressor fusions is on the expression of drug transport proteins rather than their stability or localization.

We expect that a major use of the Pdr1DBD:repressor fusions will be to analyze gene deletions affecting sensitivity to a drug of interest of the gene deletions using the yeast deletion collection. For example, genetic screens have been carried out using the yeast deletion set to identify genes required for surviving DNA damage from ionizing radiation or simple alkylating agents (Bennett et al., 2001; Chang et al., 2002; Westmoreland et al., 2004). Similar experiments using anticancer agents such as etoposide or antimetabolites such as aphidicolin require sufficient drug accumulation to elicit a biological effect. These screening experiments require alteration of a large number of strains; therefore, the con-



**Fig. 4.** Northern analysis of strains carrying pdr1DBD-fusion constructs confirms the repression of *PDR1* target genes. Northern blot analysis of *PDR5* and *YOR1* gene expression in strains carrying vector control (lane 1), pdr1DBD-GFP fusion (lane 2), *CYC8* overexpression (no fused DNA binding domain) (lane 3), pdr1DBD-CYC8 fusion (lane 4),  $\Delta pdr1$  strain (lane 5), and  $\Delta pdr1::pdr1DBD-CYC8$  fusion strain (lane 6). mRNA from the yeast actin gene was used as loading control.

struct needs to be introduced into strains by simple procedures. It would be desirable to introduce the Pdr1DBD:repressor fusion and delete the endogenous *PDR1* gene in a single step. To achieve this, we designed a construct with the *TPI1* promoter-expressing pdr1DBD-CYC8, *LEU2* as a selectable marker flanked by sequences homologous to the sequences flanking *PDR1* genomic locus. Integration of the construct simultaneously deletes the *PDR1* ORF and expresses the pdr1DBD-CYC8 fusion. We tested this construct in a series of deletions that we had found previously to confer hypersensitivity to Top2-targeting agents (Nitiss et al., 2006). Figure 7 shows the sensitivity of strains to different etoposide concentrations. The strains carry an empty vector, pdr1DBD-SIN3, pdr1DBD-CYC8, or the construct that deletes *PDR1* and also expresses pdr1DBD-CYC8 ( $\Delta pdr1::pdr1DBD-CYC8$ , *LEU2*). Figure 7 shows that the construct



**Fig. 5.** Deletion of *PDR1* enhances the sensitivity of pdr1DBD-repressor fusions to etoposide. A, cell sensitivity was carried out in a manner similar to that in the experiments shown in Fig. 1A using either a solvent control or 100  $\mu\text{g/ml}$  etoposide containing media. The strains used are indicated on the figure. The results of this experiment show that deletion of either *PDR1* or *PDR5* alone is insufficient to confer etoposide sensitivity and that strong synergistic sensitivity is seen with pdr1DBD-CYC8 and deletion of *PDR1* but not deletion of *PDR5*. For etoposide, pdr1DBD-CYC8 seems superior to pdr1DBD-SIN3. B, clonogenic survival assays were carried out using the strains indicated in the legend. Cells were treated in SD-leu medium containing the indicated concentrations of etoposide for 24 h. After incubation, aliquots were removed, diluted, and plated to SD-leu plates to determine viable titer. Wild-type cells were strain BY4741, and all strains shown (except for JN362a) are BY4741 derivatives. Survival is shown relative to the viable titer at the time of etoposide addition. Cytotoxicity is indicated in this experiment by a viability lower than 100%.



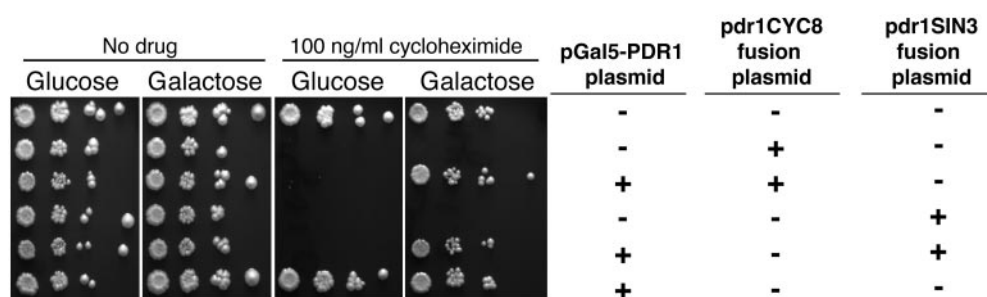
that deletes *PDR1* clearly can be used to show the etoposide sensitivity of strains lacking *MUS81*, *CTF8*, or *SAE2*. The *SAE2* deletion had the greatest effect, and sensitivity could be seen even without deletion of *PDR1*. By contrast, the sensitivity of  $\Delta mus81$  strains can be partly seen in the strain carrying *pdr1DBD-CYC8* but is most clear in the  $\Delta PDR1$  strain that carries *pdr1DBD-CYC8*.

The approach described here with *Pdr1DBD*:repressor fusions depends on the ability of the construct to enhance the accumulation of a large number of compounds with differing chemical structures. The data presented in Figs. 1 and 2 illustrate that cells carrying *pdr1DBD-CYC8* are sensitive to growth inhibition by small molecules acting against several different targets. To further test the suitability of our strategy for enhancing drug accumulation, we tested the sensitivity of a panel of 80 approved or experimental anticancer agents obtained from Discovery Microsource. This set of compounds includes DNA damaging agents, topoisomerase inhibitors, and compounds acting by unknown mechanisms such as garlicin and agents that do not damage DNA such as gossypol (Cheng et al., 2002; Qiu et al., 2002). The set of compounds also includes agents that would not act in yeast (such as nucleoside analogs) because of the absence of appropriate activating enzymes. For cells carrying only a *pYX* empty vector, 8 of 80 compounds conferred some growth inhibition. Single deletion of either *PDR1* or *PDR5* did not notably increase the number of compounds that inhibited growth. By contrast, 37 of 80 compounds showed growth inhibition in strains carrying a *PDR1* deletion along with *pdr1DBD-CYC8* (i.e., the *pdr1DBD-CYC8* construct integrated at *PDR1*). Examples of growth inhibition of five compounds along with  $\Delta pdr1$  and  $\Delta pdr5$  controls are shown in Fig. 8. These results indicate that this strategy should be useful for studying a broad range of compounds in yeast that are not readily assayable using standard approaches.

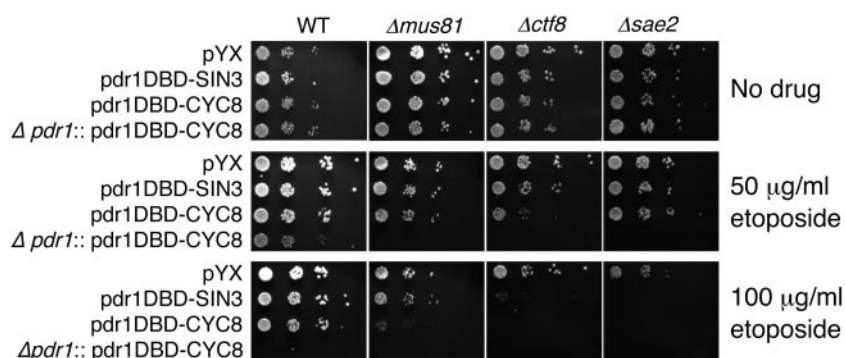
## Discussion

A wide variety of genetic tools have been developed with *S. cerevisiae* to explore fundamental aspects of cell biology in a eukaryotic system. A notable tool has been the development of a set of yeast strains that delete each of the open reading frames found in the yeast genome. This tool has been of fundamental importance for identifying genes required for a variety of basic cellular processes. For example, large numbers of genes important for surviving DNA damage, fitness under various growth conditions, and cell morphology have been identified using the yeast deletion set (Scherens and Goffeau, 2004). The deletion set has also been applied to study the action of small molecules of therapeutic interest. Several powerful approaches have been devised based on the identification of gene deletions conferring drug hypersensitivity (Baetz et al., 2004; Deng et al., 2005) or drug resistance (Huang et al., 2005). Although most of the experimental approaches with the *S. cerevisiae* deletion strains have been restricted to genes that are not essential for viability, it is also possible to include essential genes by screening for drug sensitivity (or resistance) using phenotypes observed in heterozygous diploids (phenotypes arising from haploinsufficiency) or by examining phenotypes conferred by gene overexpression. These approaches also have great potential in identifying mechanisms of action of small molecules but are limited to compounds that accumulate in yeast cells at levels that can produce significant biological effects. In this work, we describe a simple strategy applicable to large strain collections that can enhance the accumulation of diverse small molecules.

Strategies for enhancing drug accumulation in yeast can be successful, but they typically rely on introducing multiple mutations affecting efflux of small molecules and possibly small molecule influx as well (Emter et al., 2002). Although multiple mutations can be used with a small set of strains, it



**Fig. 6.** Ectopic expression of *pdr5* from a heterologous promoter restores cycloheximide resistance. Strains expressing *pdr1DBD*-repressor fusions were cotransformed with a plasmid expressing the *PDR5* gene under the control of the *GAL1* promoter. Cells were spotted on glucose or galactose plates containing 100 ng/ml cycloheximide. The plasmids carried by each strain are shown in the figure.



**Fig. 7.** Applications of the *pdr1DBD*-repressor fusions: sensitive detection of etoposide hypersensitivity in yeast deletion strains. The *pdr1DBD*-fusion constructs enhance the sensitivity of various deletion strains to etoposide. Cells carrying a *pYX* empty vector, *pdr1DBD-SIN3*, *pdr1DBD-CYC8*, or  $\Delta pdr1::pdr1DBD-CYC8$  were tested for sensitivity to etoposide. The strains used were wild-type BY4741, BY4741  $\Delta mus81$ , BY4741  $\Delta ctf8$ , or BY4741  $\Delta sae2$ . Enhanced sensitivity for a fusion construct is noted by comparing wild type with the three mutants at a fixed etoposide concentration. For example, at 50 µg/ml etoposide, enhanced sensitivity of all three mutants is seen with  $\Delta pdr1::pdr1DBD-CYC8$ . Enhanced sensitivity of the  $\Delta ctf8$  strain is also seen with *pdr1DBD-CYC8*. At 100 µg/ml etoposide, enhanced sensitivity of the  $\Delta ctf8$  and  $\Delta sae2$  strains can be clearly discerned.

is impractical to introduce more than a single mutation to enhance drug accumulation. Of the candidate single mutations available, the most commonly used are deletions of *ERG6*. This mutant has significant growth defects, is incompatible with tryptophan auxotrophy, and has very poor transformation efficiency. Neither *PDR1* nor *PDR5* single deletions gives a broad-spectrum sensitivity. As shown in Fig. 1A, the fusions described here give superior drug sensitivity compared with *PDR1* or *PDR5* deletions. The data presented here demonstrate that the pdr1-repressor fusions result in enhanced sensitivity to several model compounds. We have also examined a broad range of other agents, including other experimental anticancer agents, and have found that the repressor fusions confer hypersensitivity to agents acting against diverse cellular targets.

We also examined the ability of the DNA binding domains of other Pdr regulators to affect drug sensitivity. We constructed fusions containing the DNA binding domains of *PDR3* and *YRR1* along with the coding sequence of *SIN3*. Because the Pdr3 DNA binding domain recognizes the same nucleotide sequence as the Pdr1 DNA binding domain (Katzmann et al., 1994; Moye-Rowley, 2003), we anticipated that the pdr3DBD-Sin3 fusion would confer cycloheximide sensitivity. By contrast, Yrr1 regulates the expression of Snq2 but not Pdr5, and loss-of-function mutants of Yrr1 do not confer cycloheximide sensitivity (Moye-Rowley, 2003). As expected, the pdr3DBD-Sin3 fusion conferred cycloheximide sensitivity, whereas the yrr1DBD-Sin3 fusion did not confer cycloheximide sensitivity (A. Stepanov and J. L. Nitiss, unpublished data). Although the fusions using either the Pdr3 or Yrr1 DNA binding domains were not extensively characterized, they may be useful in studying compounds unaffected by expression of the Pdr1 repressor fusions.

We anticipated that the pdr1-repressor fusion constructs would confer dominant drug sensitivity. Although this expectation was partly correct, we found that deletion of *PDR1* along with introduction of the *PDR1* fusion resulted in

greater sensitivity than expression of the fusion in a strain carrying the wild-type *PDR1* gene. The microarray data presented in Fig. 3 also suggest that we achieve much greater repression of *PDR1*-regulated genes using the pdr1-repressor fusions when *PDR1* is also deleted. This does not make the construct more difficult to use for most applications, because some of our pdr1-repressor fusions are targeted to delete the wild-type *PDR1* gene. The pdr1 repressor is then introduced into the deletion set by mating (Tong et al., 2001). One application in which the targeting of the pdr1-repressor construct to the *PDR1* locus is impractical is introducing the construct into the set of strains in which the targeted ORF deletions are heterozygous. This set of strains is important because it includes (heterozygous) deletions of essential yeast genes. Several robust approaches have been described for transforming large numbers of strains using robotic platforms, and substantial drug sensitivity can be seen even when wild-type Pdr1 is expressed. Furthermore, there is no practical way to introduce any (recessive) mutation affecting drug accumulation into the heterozygous diploid collection.

An additional strength of our approach is the demonstration that multiple pdr1-repressor fusions are capable of repressing the expression of Pdr1-regulated genes. *SIN3* and *CYC8* share some mechanisms of gene repression but also require different complements of proteins to affect repression. The availability of two different constructs allows investigators to minimize effects that are independent of the repression of genes of the Pdr network.

We envision that the pdr1-repressor fusions can be applied to a variety of problems relating to the analysis of drug action. For example, as shown in Fig. 7, we used the pdr1-repressor fusion to test the importance of several repair genes in sensitivity to etoposide. Little sensitivity to etoposide is seen for any of the repair-deficient strains when they carry an empty vector, but sensitivity was clearly seen for both the pdr1-Sin3 and pdr1-Cyc8 fusions. Similar analyses can be performed with drugs with known targets (such as etoposide) and drugs with more poorly defined targets.

We have also demonstrated that ectopic expression of Pdr5p reverses the cycloheximide sensitivity of cells carrying a pdr1-repressor fusion. The ability to extinguish the expression of several yeast transport proteins will allow the development of yeast strains expressing heterologous drug transport proteins. This may represent a particularly efficient system for determining substrate-specificity and inhibitor profiles for transport proteins of therapeutic interest.

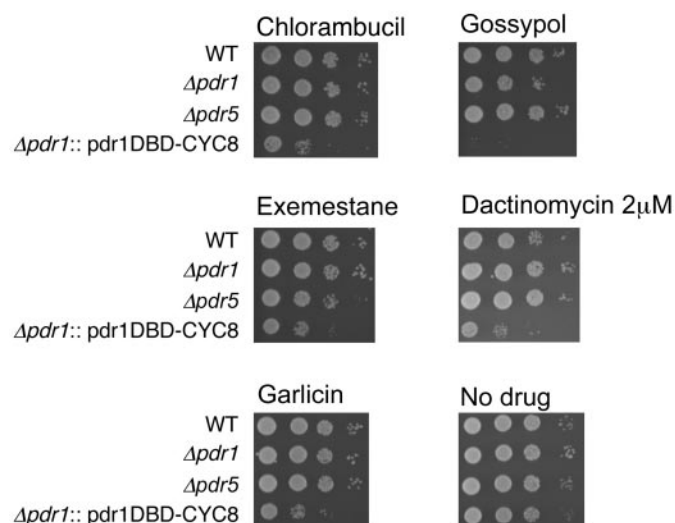
In conclusion, we have developed a novel approach to enhance drug accumulation in *S. cerevisiae*. We have demonstrated specific repression of yeast genes that are regulated as part of the Pdr1/Pdr3 network, resulting in enhanced drug accumulation and drug efficacy. This approach opens up yeast to the study of much broader range of small molecules than was possible previously.

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**Fig. 8.** The  $\Delta pdr1::pdr1DBD-cyc8$  insertion construct enhances the sensitivity of yeast to a wide range of anticancer drugs. Logarithmically growing cells were spotted onto plates after 24 h of drug treatment in liquid. An assortment of small molecules/drugs as described in the text was assessed. All drugs were tested at 250  $\mu$ M except for dactinomycin (2  $\mu$ M). Five positive compounds are illustrated. Other positive compounds are not shown, and a list of test compounds is available on request.



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