

## Invited Review

# Functional Analysis of Fungal Drug Efflux Transporters by Heterologous Expression in *Saccharomyces cerevisiae*

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**SUMMARY:** Clinically important resistance of fungal pathogens to azole antifungal drugs is most frequently caused by the over-expression of energy-dependent drug efflux pumps. These pumps usually belong to either the ATP-binding cassette (ABC) family or the Major Facilitator Superfamily (MFS) class of membrane transporter. Little is known about how these pumps work and there is an urgent need to develop pump antagonists that circumvent azole resistance. We have developed a protein hyper-expression system to facilitate functional analysis of efflux pumps based on a *Saccharomyces cerevisiae* host which has been deleted in seven major ABC transporters to reduce the background of endogenous efflux activity. Plasmid pABC3 was engineered to allow functional hyper-expression of foreign proteins in this host. The main advantages of the system include its ease of directional cloning and the use of homologous recombination to stably integrate single copy constructs into the host genome under the control of a highly active transcriptional regulator. The system has been used to clone and functionally hyper-express genes encoding drug efflux pumps from several pathogenic fungi. Furthermore, the protein hyper-expression system has been used to screen for pump inhibitors and study the structure and function of heterologous membrane proteins.

### Introduction

Opportunistic fungal infections have become a problem of increasing importance over the past two decades, paradoxically because modern medical practice has allowed the survival of debilitated patients and immunosuppressed individuals with AIDS, cancer and organ transplants. Such patients are highly susceptible to infection by opportunistic fungal pathogens such as *Candida* spp., *Aspergillus fumigatus* and *Cryptococcus neoformans*. These problems have stimulated basic and clinical research on these medically important fungi. A variety of molecular genetic techniques, including gene isolation, gene disruption, transcriptional analysis and proteomics methods, been developed and these now underpin biological studies of pathogenic fungi (1-5). Genome sequence information for several fungal pathogens has also gradually accumulated. An almost complete sequence of *Candida albicans*, the most prominent fungal pathogen, is now available (6; <http://www-sequence.stanford.edu/>

<http://candida.stanford.edu/>), as are sequences for *Candida glabrata* (7; <http://cbl.labri.fr/Genolevures/>) and *A. fumigatus* (<http://www-genome.wi.mit.edu/annotation/fungi/aspergillus/>). Although rapid molecular identification techniques and novel antifungal drugs have been developed (8), the diagnosis and treatment of fungal infections still can be problematic, and the potential for drug resistance represents a growing threat to antifungal therapy.

Current treatments for systemic fungal infections use a limited number of antifungal drugs: the polyenes (amphotericin B), the azoles (fluconazole, miconazole, itraconazole, voriconazole) and flucytosine. The recent development of echinocandin antifungals such as caspofungin and micafungin, which inhibit fungal cell wall glucan synthesis, provide additional therapeutic options for the treatment of patients with systemic *Aspergillus* and *Candida* infections. Fungicidal drugs are preferred for the treatment of systemic infections in immunocompromised patients. However, the existing fungicidal drugs like amphotericin B cause significant side effects. Fluconazole, although only fungistatic, has been widely used in patients at high-risk of *Candida* infections without causing any major side effects. In cases of treatment failure, often azole-resistant clinical strains were isolated from the patients (9-12).

In this review, we describe a recently developed protein hyper-expression system utilizing a *Saccharomyces cerevisiae* host strain deleted in seven major ATP-binding cassette (ABC) transporters which thus has a reduced background of

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endogenous efflux activity. The expression system not only facilitates the functional analysis of heterologous proteins, including fungal multidrug efflux pumps responsible for high level azole resistance, but also provides the tools needed to screen for pump inhibitors. Such inhibitors can, for instance, chemosensitize cells to pump substrates like fluconazole.

### Azole resistance in pathogenic fungi

Several different mechanisms are known to contribute to the decreased susceptibility of azole-resistant clinical isolates (12-15). Changes in the expression, and mutations, of the ergosterol biosynthetic enzyme lanosterol 14 $\alpha$ -demethylase, the main target of azole drugs, decrease the susceptibility of fungal cells to these drugs. Other mechanisms can lower the internal drug concentration in fungal cells so that ergosterol biosynthesis is no longer inhibited. While clinical isolates with high-level azole resistance have often developed multiple mechanisms that contribute to the resistance (16, 17), the main cause is usually the over-expression of efflux pumps (18,19). These pumps are principally present in the plasma membrane of fungal cells and extrude drugs from the cells. Similar mechanisms of drug efflux have been characterized in many prokaryotic and eukaryotic cells, for example the bacterial tetracycline resistance pump and the mammalian P-glycoprotein. Genes encoding drug efflux pumps in several pathogenic fungi (Table 1) have been implicated in drug resistance, primarily through the use of gene disruption techniques and transcriptional analysis (20-34). Two different types of efflux pump transporters have been detected; they are ABC transporters and Major Facilitator Superfamily (MFS) transporters. The ABC transporters efflux a wide range of structurally unrelated compounds at the expense of ATP hydrolysis. The MFS transporters have narrow substrate specificity and are thought to use the proton motive force of

the plasma membrane as a source of energy.

The rising incidence of infections caused by non-*albicans* *Candida* spp. such as *C. glabrata* and *C. krusei* is of particular concern (35), because *C. glabrata* and *C. krusei* appear to be innately resistant to moderate (<64  $\mu$ g/ml) and significant (>64  $\mu$ g/ml) levels of fluconazole, respectively. We have shown that fluconazole exposure can rapidly induce the expression of both the ABC transporter CgCdr1p and the drug target lanosterol 14 $\alpha$ -demethylase in *C. glabrata* (36). This property may explain why *C. glabrata* infections emerged after azole antifungal drugs were widely used to treat systemic fungal infections (37).

### Expression of multidrug efflux pumps in *S. cerevisiae*

We have developed a convenient system for the heterologous hyper-expression of ABC transporters, and other membrane proteins, in the yeast *S. cerevisiae* AD1-8u<sup>-</sup> (38, 39). This has allowed us to characterize the structure and function of some of these proteins in detail. The strain AD1-8u<sup>-</sup> is deleted in seven major ABC transporters, Pdr5p, Pdr10p, Pdr11p, Pdr15p, Snq2p, Yor1p and Ycf1p, which makes this host exquisitely sensitive to azole antifungals and a range of other xenobiotics (38,39). Constitutive high-level expression of the heterologous protein is driven from the highly active *PDR5* promoter due to the presence of the mutant transcription factor Pdr1p which contains the *pdr1-3* the gain-of-function mutation (38). This transcriptional regulator affects a number of genes in addition to *PDR5*, ensuring that the hyper-expressed protein is trafficked to the plasma membrane.

Our convenient and versatile system for cloning and heterologous expression has been obtained by improving the vector pSK-PDR5PPUS (Fig. 1A) (38,39). The derivative vector pABC3 (Fig. 1B) contains an extra unique *EcoRI* site downstream of the *URA3* marker and a second *AseI* site just downstream of the transformation-cassette. The transformation cassette in pABC3 consists of the *PDR5* promoter, a multiple cloning site, the *PGK1* terminator, the *URA3* marker and 277 bp of the 3' end of the *PDR5* open reading frame. The rare 8 bp *PacI* and *NotI* restriction sites in the vector's multiple cloning site make it possible to directionally clone almost any gene of interest. The resulting transformation-cassette can be excised with a single 8 bp-recognizing restriction enzyme (*AseI*) and used to transform strain AD1-8u<sup>-</sup> to Ura<sup>+</sup>. The linear transformation-cassette is stably integrated at the chromosomal *PDR5* locus via a double homologous crossover event and can be independently selected using the downstream *URA3* marker. Using this system, we have successfully introduced ABC and MFS transporters from *C. albicans* (39), *C. glabrata* (40) and *C. neoformans* (unpublished results) as well as human P-glycoprotein (unpublished results) into AD1-8u<sup>-</sup>. We could detect the expression of *C. albicans* Cdr1p, Mdr1p and Erg11p in plasma membranes from *S. cerevisiae* AD1-8u<sup>-</sup> transformants (Fig. 2). Unlike other membrane protein expression systems which usually require the use of antibodies to detect expression, Cdr1p was detected by coomassie staining as an intense 170 kDa band and was present at levels of >20% of plasma membrane preparation. No over-expressed protein was detected in the membranes of parent strain AD1-8u<sup>-</sup> when the empty cassette was integrated into the *PDR5* locus (Fig. 2, lane 2). The ~60 kDa Erg11p and Mdr1p bands were also detected by coomassie staining (Fig. 2, lanes 5 and 6) and the numbers of each molecule produced appeared

Table 1. Drug efflux pumps from pathogenic fungi implicated in drug resistance

| Fungus                 | Efflux pump gene                        | Type of transporter | Reference |
|------------------------|---|---------------------|-----------|
| <i>C. albicans</i>     | <i>CDR1</i>                             | ABC                 | 20        |
|                        | <i>CDR2</i>                             | ABC                 | 21        |
|                        | <i>MDR1</i> ( <i>BEN</i> <sup>R</sup> ) | MFS                 | 22        |
|                        | <i>FLU1</i>                             | MFS                 | 23        |
| <i>C. glabrata</i>     | <i>CDR1</i>                             | ABC                 | 24        |
|                        | <i>PDH1</i>                             | ABC                 | 25        |
| <i>C. dubliniensis</i> | <i>CDR1</i>                             | ABC                 | 26        |
|                        | <i>CDR2</i>                             | ABC                 | 26        |
|                        | <i>MDR1</i>                             | MFS                 | 26        |
| <i>C. krusei</i>       | <i>ABC1</i>                             | ABC                 | 27        |
|                        | <i>ABC2</i>                             | ABC                 | 27        |
| <i>C. neoformans</i>   | <i>CneMDR1</i>                          | ABC                 | 28        |
|                        | <i>CnAFR1</i>                           | ABC                 | 29        |
| <i>A. fumigatus</i>    | <i>AfuMDR1</i>                          | ABC                 | 30        |
|                        | <i>AfuMDR2</i>                          | ABC                 | 30        |
|                        | <i>AfuMDR3</i>                          | MFS                 | 31        |
|                        | <i>AfuMDR4</i>                          | ABC                 | 31        |
| <i>A. flavus</i>       | <i>AflMDR1</i>                          | ABC                 | 30        |
| <i>A. nidulans</i>     | <i>atrA</i>                             | ABC                 | 32        |
|                        | <i>atrB</i>                             | ABC                 | 32        |
|                        | <i>atrC</i>                             | ABC                 | 33        |
|                        | <i>atrD</i>                             | ABC                 | 34        |

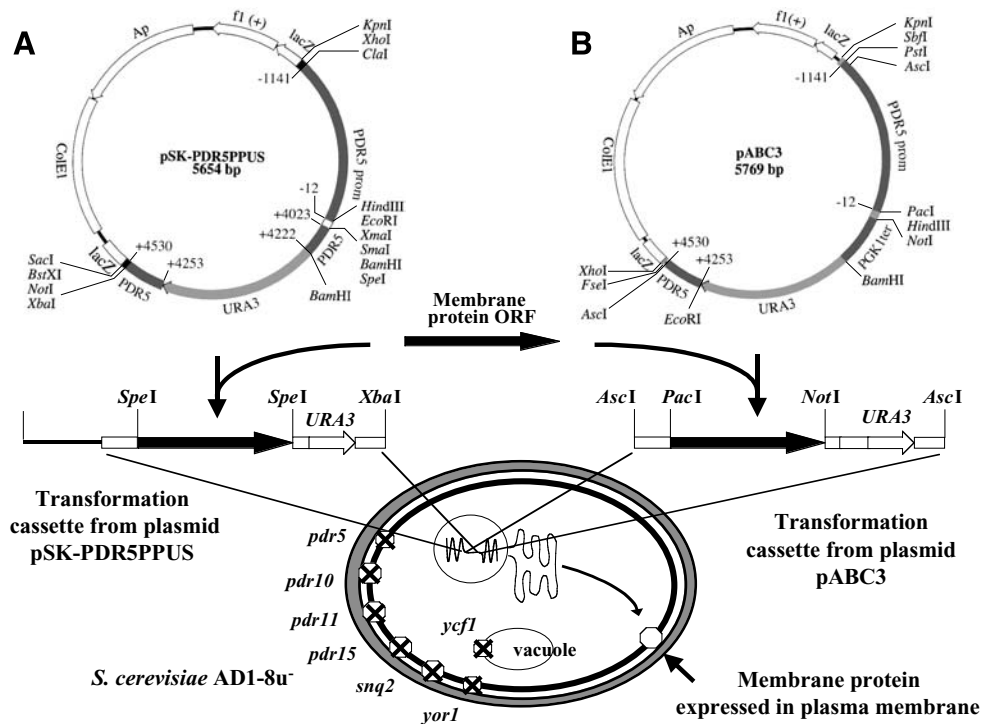


Fig. 1. Development of the vector pSK-PDR5PPUS (A) into the vector pABC3 (B) and the strategy for the cloning of ORFs into pSK-PDR5PPUS or pABC3. The transformation cassette of pSK-PDR5PPUS-ORF or pABC3-ORF integrates into the chromosomal *PDR5* locus of AD1-8u<sup>-</sup> via a double homologous cross-over. Hyper-expression of the ORF is driven by the constitutively transcribed *PDR5* promoter under the influence of the Pdr1p transcription factor containing the *pdr1-3* gain-of-function mutation.

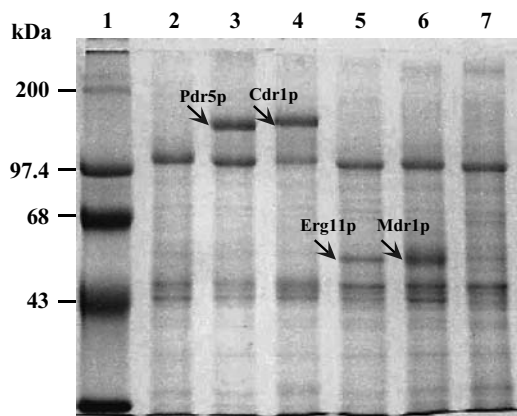


Fig. 2. Hyper-expression of different classes of heterologous membrane proteins in the *S. cerevisiae* host strain AD1-8u<sup>-</sup>. Plasma membrane proteins (30  $\mu$ g) were separated by SDS-PAGE in an 8% polyacrylamide gel. Lane 1, molecular weight markers. Lanes 2 to 7 were loaded with plasma membrane proteins isolated from the following strains: lane 2, AD-pABC3; lane 3, AD-pABC3-ScPDR5; lane 4, AD-pABC3-CaCDR1; lane 5, AD-pABC3-CaERG11; lane 6, AD-pABC3-CaMDR1 and lane 7, AD1-8u<sup>-</sup>. Arrows indicate the protein bands hyper-expressed in lanes 3 to 6.

comparable to those for the 100 kDa plasma membrane proton-pumping ATPase, the dominant protein band in yeast plasma membrane preparations. The identity of Cdr1p and Mdr1p proteins could be confirmed by using specific antibodies. These and other hyper-expressed protein bands can also be identified by MALDI-TOF mass spectrometry of tryptic fingerprints (36). Both the original and our improved hyper-expression system have been used to show that green fluorescent protein-tagged Cdr1p (Cdr1p-GFP) was fully

functional and properly located in the plasma membrane (unpublished data, 41).

### Functional analysis of efflux pumps

Strains that functionally hyper-express efflux pumps in the AD1-8u<sup>-</sup> host provide an excellent system for comparative studies of drug pumps. In our laboratories, we routinely use *S. cerevisiae* expressing homologous or heterologous pumps to determine the MIC values of drugs for these strains, evaluate pump substrate specificity, estimate uptake and efflux activity with fluorescent substrates like rhodamine 6G, and measure the nucleoside triphosphatase (NTPase) activity of hyper-expressed pumps. For example, we compared the MIC values of fluconazole for *S. cerevisiae* strains expressing *C. albicans* *CDR1* and *CDR2* (both genes encode ABC transporters), *MDR1* (encoding an MFS transporter) or *ERG11* (encoding lanosterol 14  $\alpha$ -demethylase). The *CDR1*-expressing strain gave the highest fluconazole MIC value (400  $\mu$ g/ml), followed by the *CDR2*- (80  $\mu$ g/ml), *MDR1*- (60  $\mu$ g/ml) and *ERG11*-expressing strains (2  $\mu$ g/ml), whereas the MIC of AD1-8u<sup>-</sup> was 0.6  $\mu$ g/ml. This pattern of MIC values matches the pattern of susceptibilities of azole-resistant *C. albicans* clinical isolates over-expressing individual genes and, as expected, only Cdr1p and Cdr2p showed broad substrate specificities. Their hyper-expression conferred resistance to the azole antifungals fluconazole, ketoconazole and itraconazole, and to structurally and functionally unrelated compounds such as cycloheximide, rhodamine 6G and cerulenin, but not to the polyene antibiotic amphotericin B and nystatin. In contrast, the *MDR1*-expressing strain was resistant only to fluconazole, consistent with the narrow substrate specificity expected of this MFS

transporter (42), while the Erg11p hyper-expressing strain conferred resistance only to the azole drugs that target this enzyme. The glucose-dependence of the rhodamine 6G efflux activity of the Cdr1p and Cdr2p pumps was used to confirm that these ABC transporters need ATP hydrolysis to function. Rhodamine 6G was not extruded from the parent AD1-8u<sup>-</sup> strain even in the presence of glucose. These studies are providing us with the tools needed to explore the function of different drug pumps and their alleles in clinical isolates that have developed drug resistance in response to fluconazole treatment and prophylaxis.

*CneMDR1* is an ABC transporter expressed by the medically important fungal pathogen *Cryptococcus neoformans*. *CneMDR1* was cloned from cDNA and expressed in the *S. cerevisiae* hyper-expression system. The *CneMDR1* hyper-expressing strain showed similar glucose-dependent rhodamine 6G efflux activity to strains hyper-expressing *CaCDR1* and *CgCDR1*, an oligomycin-sensitive nucleotide triphosphatase activity but differential sensitivities to azole drugs and some other xenobiotics (unpublished results). Our *S. cerevisiae* constructs are therefore allowing us to elucidate the specificity and regulation of the heterologously expressed *Cryptococcus* efflux pump in ways that are not yet possible with the donor organisms.

We have isolated plasma membranes from *S. cerevisiae* strains that heterologously hyper-express ABC transporters from various fungi and have determined the NTPase activity of each ABC transporter. The efflux pumps hydrolyse CTP, GTP and UTP as well as ATP (39,40) but we have yet to demonstrate which nucleoside determines pump activity. Studies of the closely related *S. cerevisiae* Pdr5p pump show that while the enzyme's NTPase activity has broad specificity, pump activity requires ATP (43). Studies with isolated secretory vesicles, obtained by blocking their transit to the plasma membrane using an AD1-8u<sup>-</sup> derivative strain containing the temperature-sensitive *sec6-4* mutation, or with purified plasma membranes will allow advanced studies of pump biochemistry.

### Post-translational regulation of drug efflux pumps

The transcriptional regulation of yeast ABC transporter genes involved with drug efflux activity has been extensively studied (for reviews, see 44 and 45), but little is known about the effects on the function of fungal drug efflux pumps caused by post-translational modifications such as phosphorylation, glycosylation and ubiquitination. In fungi, there are few reports on the phosphorylation of drug ABC transporters. The phosphorylation of a PKA site was found to be essential for cadmium efflux by *S. cerevisiae* Ycf1p (46). Phosphorylation, regulated by type 2A phosphatase Sit4p, was suggested to be important for the activity of *Kluyveromyces lactis* Pdr5p (47), and the phosphorylation of *S. cerevisiae* Pdr5p, Snq2p, and Yor1p has also been reported (48). Phosphorylation by casein kinase I was required for the stability of Pdr5p in the plasma membrane (48). Although the *C. glabrata* Cdr1p and Pdh1p pumps are close homologues of Pdr5p, both lack the sites equivalent to the casein kinase I site found in Pdr5p. Functional hyper-expression of *C. glabrata* Cdr1p and Pdh1p in AD1-8u<sup>-</sup> has demonstrated that both pumps were phosphorylated in vivo in a glucose-dependent manner (40). Although phosphorylation of the pumps may occur at multiple sites and be catalysed by several kinases, both Cdr1p and Pdh1p were phosphorylated by mechanism(s) that

Table 2. Applications of the *S. cerevisiae* hyper-expression system

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|---|
| • Characterization of drug efflux transporters  |
| Substrate specificity   |
| Rhodamine 6G pumping  |
| NTPase activity   |
| Membrane protein analysis by:   |
| SDS-PAGE, Western blotting, MALDI-TOF MS,   |
| affinity purification   |
| Post-translational modification detection and analysis:   |
| Phosphorylation, glycosylation, ubiquitination, etc.  |
| Site-directed mutagenesis, structural and functional analysis   |
| • Screening and counterscreening  |
| Identification of pump inhibitors (chemosensitizers)  |
| Identification of compounds which are not pump substrates   |
| Evaluation of the effects of known drug-resistance mechanisms   |
| • The functional heterologous expression of additional classes of membrane protein (e.g., human P-glycoprotein) for biochemical, structural and screening applications. |

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appear to involve protein kinase A isoforms (40,49). The phosphorylation of Cdr1p in glucose-starved cells was increased by the addition of glucose, as in the case of Pdh1p, but this required mM rather than  $\mu$ M glucose concentrations. The differential phosphorylation of these transporters may contribute to differences in enzyme activities and the drug efflux specificities of Cdr1p and Pdh1p (40,49). The *S. cerevisiae* hyper-expression system provides an invaluable tool for analytical and functional studies of the post-translational phosphorylation of fungal ABC transporters and could be applied to other proteins and other forms of post-translational modification (Table 2).

### Screening of inhibitors of fungal drug efflux pumps

Because fungal cells are eukaryotes, it is difficult to develop drugs that inhibit fungal cells selectively without affecting their mammalian counterparts. The recently marketed echinocandin antifungal agents, such as caspofungin and micafungin, are improved drugs in this sense, because their target molecules are thought to be cell wall biosynthetic enzymes, which do not exist in mammalian cells (50). They will partially satisfy the need to combat the increasing frequency of systemic fungal infections caused by opportunistic fungal pathogens like *A. fumigatus*, which is intrinsically resistant to fluconazole. However, both caspofungin and micafungin cannot be administered orally and are therefore reserved for the treatment of systemic infections. There is also the need to overcome the problem of resistance to the widely used, well-tolerated, and bioavailable azole drugs. One way to bypass azole resistance is to find compounds that inhibit drug efflux activity. Since the efflux pumps are not essential enzymes, except in the presence of xenobiotics, specific pump inhibitors should not be toxic when used alone. However, the use of such compounds, in combination with fluconazole, is expected to chemosensitize resistant cells and thus potentiate the efficacy of the azole drug. There have been several attempts to screen for inhibitors of drug efflux pumps using fluconazole-resistant *C. albicans* clinical isolates or *S. cerevisiae* strains hyper-expressing *C. albicans* efflux pumps. The immunosuppressive agents FK506 and cyclosporine have been shown to chemosensitize azole-resistant *C. albicans* cells to fluconazole (51-53). We have shown that FK506 plus fluconazole inhibited the growth

of a Pdr5p hyper-expressing, fluconazole-resistant *S. cerevisiae* strain. Inhibitory effects on rhodamine 6G efflux and the NTPase activity of drug pumps were also detected (unpublished results). As systemic fungal infections including candidiasis are opportunistic fungal infections in immunocompromised hosts, an immunosuppressant is probably not the ideal compound to combine with fluconazole.

The milbemycins, a group of 16 membered ring macrolides, have been reported as potent inhibitors of the Cdr1p pump in *C. albicans* and shown to potentiate the antifungal activity of fluconazole (53). As milbemycins are not immunosuppressive agents, we have tested the antifungal activity of a panel of milbemycins in combination with fluconazole. We found that the milbemycins did not inhibit the growth of *S. cerevisiae* strains hyper-expressing multidrug efflux pumps, and that *CDR1*- or *CDR2* hyper-expressing strains were strongly sensitized to fluconazole by several of the milbemycins. An *MDR1* hyper-expressing fluconazole-resistant strain was not affected by the combination of fluconazole and milbemycins and some of the milbemycins inhibited Cdr1p- and Cdr2p-ATPase but not plasma membrane H<sup>+</sup>-ATPase activity (unpublished results). These observations suggest that some milbemycins may inhibit the ABC transporter specifically and thereby potentiate azole antifungal activity. The milbemycins are related to drugs used as antihelminthics. They are thus likely to be well tolerated by mammals and may have considerable potential as small molecule drugs.

We have also screened a unique combinatorial peptide library for compounds that chemosensitize efflux pump hyper-expressing *S. cerevisiae* strains to fluconazole and inhibit pump ATPase activity in vitro. The library consists of 1.89 million different octapeptides synthesized from D-amino acids. We identified KN20, a potent noncompetitive efflux pump inhibitor, which enhanced the efficacy of azole drugs against model and pathogenic drug-resistant fungi (54). Overall, the *S. cerevisiae* hyper-expression system provides an excellent platform for screening and evaluating pump inhibitors.

The expression system can also provide counterscreens to identify and eliminate compounds that are transporter substrates, and to identify drug candidates that are not. This capability may be extremely valuable for the pharmaceutical industry, allowing a cost-effective focus on new chemical entities that are not susceptible to drug resistance mediated by efflux pumps. In addition, with the ability to mutate open reading frames and incorporate affinity tags, our hyper-expression system allows the high-level functional expression and rapid purification of membrane proteins engineered to reduce protein microheterogeneity, as required for protein crystallization and crystallography. We therefore expect that the hyper-expression system will make a major contribution to the structural and functional analysis of not only fungal ABC and MFS transporters and lanosterol 14 $\alpha$ -demethylase, but also many other membrane proteins.

### Conclusion

We have developed a versatile protein hyper-expression system to facilitate functional analysis of efflux pumps that is based on a *S. cerevisiae* host strain deleted in seven major ABC transporters. The main advantages of the system are its cloning efficiency and the use of homologous recombination to integrate stable, single copy, constructs into the host

genome under the control of a highly active transcriptional regulator. The expression system has been used to clone and functionally express, in their correct context, genes encoding drug efflux pumps from several pathogenic fungi. The protein hyper-expression system allows the comparative analysis of multidrug efflux pumps of pathogenic fungi and the screening of pump inhibitors (Table 2). We also predict that the system will provide powerful tools for the structural and functional study of many classes of membrane protein.

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