ERG6 and *PDR5* regulate small lipophilic drug accumulation in yeast cells via distinct mechanisms

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Abstract Diagnosis and circumvention of multi-drug resistance requires an understanding of the underlying cellular mechanisms. In the model organism *Saccharomyces cerevisiae*, deletions of *PDR5* or *ERG6* increase sensitivity to many small lipophilic drugs. Pdr5p is a plasma membrane ATP-binding cassette transporter that actively exports drugs, thereby lowering their intracellular levels. The mechanism by which *ERG6*, an enzyme in sterol biosynthesis, affects drug accumulation is less clear. We show here that *ERG6* limits the rate of passive drug diffusion across the membrane, without affecting Pdr5p-mediated drug export. Consistent with their action by distinct mechanisms, *PDR5* and *ERG6* effects on drug accumulation are additive.

Key words: Drug resistance; ATP-binding cassette transporter; Sterol; Small lipophilic molecule transport; Yeast

1. Introduction

Multi-drug resistance is a common problem in the chemotherapeutic treatment of cancer and microorganism-caused disease [1,2]. Among the several underlying causes, mechanisms that affect intracellular drug levels are prominent [1]. Increased activity of exporters with broad substrate specificity leads to reduced intracellular accumulation and potency of drugs [3–7]. Changes in the intracellular pH lead to reduced drug sequestration [8]. Defects in transporter-mediated uptake contribute to drug resistance in trypanosomes [9]. Finally, changes in the lipid composition of the plasma membrane have been suggested to affect drug accumulation (reviewed in [10]).

The non-pathogenic yeast *Saccharomyces cerevisiae* has served as a model organism for the genetic identification of multi-drug resistance mechanisms [4]. Wild-type yeast are naturally resistant to many drugs that are toxic to higher eukaryotes, suggesting that some resistance mechanisms are constitutively active. One such mechanism is ATP-binding cassette (ABC) transporter-mediated drug efflux. The yeast genome encodes 22 putative ABC transporters. Four of these (Pdr5p, Yor1p, Snq2p and Ycf1p), protect yeast from a wide variety of drugs and confer increased resistance when overexpressed (reviewed in [6]). Other loci contributing to drug resistance include the transcriptional regulators Pdr1p, Pdr3p, Yap1p and Sin4p, which control the expression of ABC transporters or other resistance determinants [4,11]. Mutations in proteins that affect lipid metabolism suggest yet another mechanism of drug resistance. Yeast lacking the phosphatidylinositol transfer protein Sec14p homologues Pdr16p and Pdr17p [12,13], or the C-24 sterol methyltransferase Erg6p, are hypersensitive to many drugs [14–21]. Mutations in *PDR16* and *PDR17* lead to an increase in passive drug uptake into cells [12]. The hypersensitivity of yeast lacking Erg6p, an enzyme that acts in the late steps of ergosterol biosynthesis, has been attributed to membrane 'permeability' changes that enable the uptake of small molecules [14,19]. Currently, it is unclear whether this is due to changes in passive diffusion across the membrane or in active transport systems for these molecules. In support of the latter mechanism, the ABC transporter Pdr5p has been reported to display reduced export activity in *erg6Δ* cells [21].

S. cerevisiae has also been a model system for genetic studies of mammalian steroid hormone signaling [22]. The small lipophilic steroid hormones bind and activate intracellular receptors, which are hormone-dependent transcription factors. Although steroid receptors are not naturally present in yeast, this organism provides the basic machinery necessary for their function [22]. Genetic screens in yeast have identified *PDR5* and *ERG6* as genes that inhibit the transcriptional response to hormone, by preventing the intracellular accumulation of hormone and subsequent receptor activation [23,24]. The ability of Pdr5p to actively export steroids explains the increased hormone accumulation in *pdr5Δ* cells [23,25,26]. The mechanism by which defects in *ERG6* increase intracellular hormone levels is less clear.

In this study, we address the mechanism by which Erg6p affects sensitivity to steroids and other drugs. We show that Pdr5p is functional in *erg6Δ* cells, that *PDR5* and *ERG6* have additive effects on drug resistance, and that Erg6p affects passive diffusion of drugs across the membrane.

2. Materials and methods

2.1. Yeast strains

Strains used in this study are derived from YPH501 [27]. YNK310 (*ERG6*, *PDR5*, *leu2::GRE-lacZ*), YNK410 (*ERG6*, *pdr5::GRE-lacZ*, *leu2::GRE-lacZ*) [28], YNK589 (*erg6::HIS3*, *PDR5*, *leu2::GRE-lacZ*) and YNK591 (*erg6::HIS3*, *pdr5::GRE-lacZ*, *leu2::GRE-lacZ*) carry integrated glucocorticoid-responsive *LacZ* reporters [28], as indicated. *ERG6* was disrupted with a polymerase chain reaction

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(PCR)-amplified *erg6::HIS3* fragment [24]. Gene integrations, disruptions and segregations were confirmed by PCR analysis of genomic DNA. Cells carried a *TRP1*, rat glucocorticoid receptor expression plasmid (pRS314-GN795 or pG1-N795 in Fig. 1B) [23]. In addition, YNK310 and YNK410 carried the *HIS3* expression plasmid pRS313, so as to have the same auxotrophic requirements as YNK589 and YNK591 [27]. Cells were grown in standard rich media or synthetic selective media (SD) lacking tryptophan and histidine.

2.2. β-Gal assays

Quantitative β-gal assays were carried out in 96-well microtitre plates. Yeast grown overnight were diluted 1:20 in SD media containing hormone or carrier ethanol, grown for 16 h at 30°C, permeabilised with 5% (3-[(3-cholamidopropyl)dimethylammonio]-1-propane-sulfonate) and assayed for β-gal activity [22].

2.3. Cycloheximide sensitivity

Cells grown overnight were diluted 1:15 in SD media containing 0, 1.5, 3, 6, 12, 25, 50, 100, 200, 400 or 800 ng/ml of cycloheximide, in 96-well microtitre plates. After growth for 16 h at 30°C, cell density was measured as A_{600}.

2.4. Western analysis

Protein extracts from cells grown in SD media to an A_{600} of 0.7–0.9, were resolved in sodium dodecyl sulphate–polyacrylamide gels, and transferred to nitrocellulose membranes. Pdr5p and CPY were detected with the corresponding rabbit polyclonal antibodies [29,30].

2.5. Rhodamine accumulation, uptake and efflux

To determine steady-state intracellular rhodamine 6G levels, exponentially growing cells were incubated with 5 μM rhodamine 6G in SD media for 1 h at 30°C [28]. Cells were then analysed by flow cytometry using a FACScan (Becton Dickinson) and the Cellquest software. Rhodamine efflux was determined as described by Kolaczkowski et al. [26] with minor modifications. Exponentially growing yeast (1.4×10^8) were washed, resuspended in 50 mM HEPES, pH 7, 5 mM 2-deoxy-D-glucose and 5 μM rhodamine 6G, and incubated for 2 h at room temperature. De-energised, rhodamine-loaded cells were washed once in 50 mM HEPES pH 7, and divided into two: one part was resuspended in 5 ml 50 mM HEPES pH 7 (no energy) and the other part in 5 ml of the same buffer containing 1 mM glucose (+energy). After 15 min at 30°C, cells were removed by centrifugation, and the amount of rhodamine 6G released into the media was determined by fluorescence measurement using a Jasco FP-777 spectrofluorometer, an excitation wavelength of 529 nm (1.5 nm slit) and an emission wavelength of 553 nm (3 nm slit). Measurements of rhodamine uptake were according to van den Hazel et al. [12]. Yeast (1.4×10^8) grown at 30°C in SD media (A_{600} of 0.4–0.6) were depleted of energy by incubation at 30°C for 2.5 h in energy-depletion buffer (50 mM HEPES pH 7, 1 μM antimycin A, 5 mM 2-deoxy-D-glucose). De-energised cells were collected, resuspended in 1 ml energy-depletion buffer containing 5 μM rhodamine 6G, and incubated at 30°C. Aliquots taken at different times were washed, resuspended in 50 mM HEPES pH 7, and subjected to fluorescence measurements as described above. Cell fluorescence background, measured in parallel incubations in the absence of rhodamine 6G, was subtracted from fluorescence values in the presence of the drug.

3. Results

3.1. Pdr5p is active in erg6Δ cells

The drug hypersensitivity of *erg6Δ* cells could be due to a decreased activity of membrane proteins that efflux the drugs [21]. To evaluate the function of active drug-efflux systems, we compared the activity of the endogenous yeast transporter Pdr5p in *erg6Δ* to that in *ERG6* cells. Pdr5p activity was assessed by measuring its ability to regulate the intracellular accumulation of three known Pdr5p substrates: dexamethasone (dex), cycloheximide, and rhodamine 6G, i.e. drugs with distinct structures and cellular targets.

Dex binds and activates the intracellular glucocorticoid receptor, which is a ligand-regulated transcription factor. In yeast that express the mammalian glucocorticoid receptor and carry a glucocorticoid-inducible β-gal reporter, the intracellular concentration of dex determines the percentage of activated receptor molecules, and hence the production of β-gal [22–24]. Pdr5p-mediated export decreases intracellular dex levels, and thereby compromises β-gal induction [23]. A comparison of Pdr5p activity in *erg6Δ* and *ERG6* cells demonstrated that the transporter was active in both strains (Fig. 1). In *erg6Δ* cells, the presence of Pdr5p rendered cells resistant to dex, shifting the dex concentration required to induce half the maximal response (~2000 β-gal units) from $\sim10^{-6}$ to $\sim10^{-4}$ M (compare *erg6Δ,pdr5Δ* to *erg6Δ,PDR5* in Fig. 1A). Pdr5p conferred a similar resistance to dex in *ERG6* cells; comparable β-gal levels were induced by $\sim2\times10^{-6}$ M dex in *ERG6,pdr5Δ* and $\sim10^{-4}$ M dex in *ERG6,PDR5* cells (Fig. 1A). In conclusion, Pdr5p activity was independent of *ERG6* function.

ERG6 itself caused a shift in hormone responsiveness. Lack of Erg6p increased sensitivity to dex by ~10-fold in both *PDR5* and *pdr5Δ* cells (Fig. 1A). Importantly, the effects of *PDR5* and *ERG6* on hormone responsiveness were additive, with the double *erg6Δ,pdr5Δ* yeast showing the highest sensi-

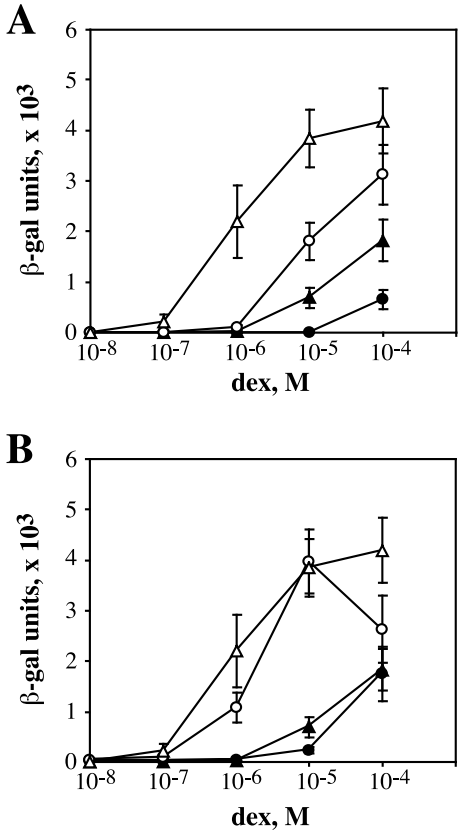

Fig. 1. Pdr5p confers resistance to dex in *erg6Δ* cells as efficiently as in *ERG6* cells. Yeast strains YNK310 (*ERG6,PDR5*) (●), YNK410 (*ERG6,pdr5Δ*) (○), YNK589 (*erg6Δ,PDR5*) (▲) and YNK591 (*erg6Δ,pdr5Δ*) (△) expressing the glucocorticoid receptor were treated with the indicated dex concentrations for 16 h and assayed for β-gal activity. The receptor was expressed from a low copy plasmid (pRS314-GN795) in all four strains (A), or from the low copy plasmid in *erg6Δ* and a high copy plasmid (pG1-N795) in the *ERG6* cells (B). The transcriptional activity of a hormone-independent glucocorticoid receptor variant was not affected by Pdr5p or Erg6p [23,24]. Data are the mean and range of duplicates from three independent experiments.

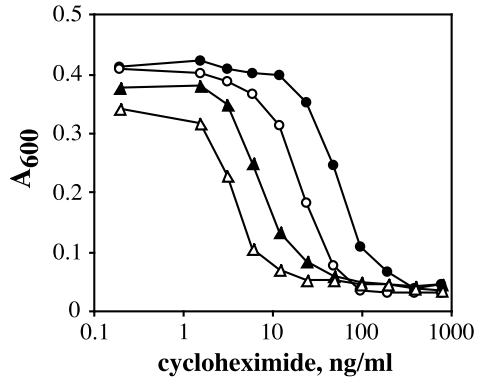

Fig. 2. Pdr5p confers resistance to cycloheximide in both *ERG6* and *ergΔ6* cells. Yeast strains YNK310 (*ERG6*,*PDR5*) (●), YNK410 (*ERG6*,*pdr5Δ*) (○), YNK589 (*erg6Δ*,*PDR5*) (▲) and YNK591 (*erg6Δ*,*pdr5Δ*) (△) were grown in selective media containing the indicated cycloheximide concentrations. The optical densities of the cell cultures (A_{600}) after 16 h of growth were plotted against the cycloheximide concentration. Data are the average of duplicates from at least three independent experiments.

tivity to dex. Because of the *ERG6* effect on hormone responsiveness, the regulatory effect of Pdr5p was assessed over different dex concentrations in *ERG6* and *erg6Δ* cells in the experiment of Fig. 1A. To evaluate Pdr5p activity over similar dex concentrations, we altered the assay conditions. We increased the levels of glucocorticoid receptor expressed in the *ERG6* cells, using a high instead of low copy receptor expression plasmid. The increase in receptor levels led to the induction of β-gal at lower hormone concentrations (compare *ERG6* cells in Fig. 1B with the same cells in Fig. 1A). Under these conditions, Pdr5p expression caused again comparable shifts to the concentrations of dex required to induce β-gal in *erg6Δ* and *ERG6* cells, demonstrating clearly that Pdr5p is similarly active in the two strains (Fig. 1B).

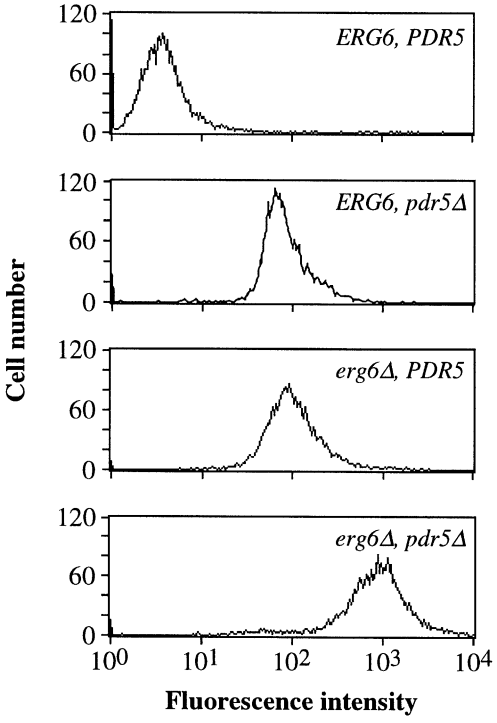

Fig. 3. Pdr5p decreases the accumulation of rhodamine 6G in both *ERG6* and *ergΔ6* cells. Yeast strains YNK310 (*ERG6*,*PDR5*), YNK410 (*ERG6*,*pdr5Δ*), YNK589 (*erg6Δ*,*PDR5*) and YNK591 (*erg6Δ*,*pdr5Δ*) were incubated with 5 μM rhodamine 6G for 1 h. Rhodamine 6G accumulation in cells was determined by flow cytometry. Data are from one experiment and are representative of three independent experiments.

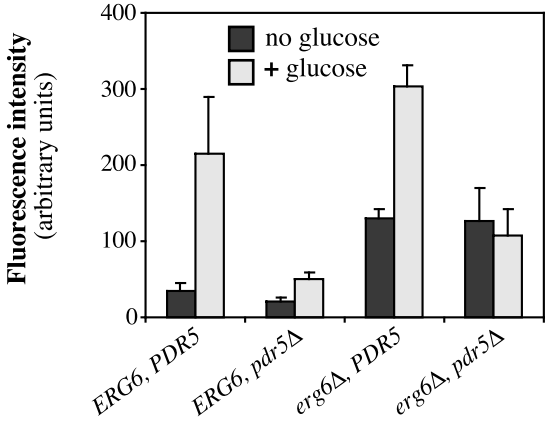

Fig. 4. Pdr5p-mediated efflux of rhodamine 6G is similar in *ERG6* and *erg6Δ* cells. Yeast strains YNK310 (*ERG6*,*PDR5*), YNK410 (*ERG6*,*pdr5Δ*), YNK589 (*erg6Δ*,*PDR5*) and YNK591 (*erg6Δ*,*pdr5Δ*) were depleted of energy and pre-loaded with rhodamine 6G for 2 h. Rhodamine 6G released into the media during 15 min at 30°C, in the absence (dark bars) or presence (light bars) of energy (glucose), was measured as described in Section 2. Data are the average from two independent experiments.

To determine the export efficiency of Pdr5p for a second substrate, we assessed the effect of Pdr5p on cell growth in increasing concentrations of the translation inhibitor cycloheximide. A comparison of *PDR5* to *pdr5Δ* cells showed that Pdr5p conferred similar increases in resistance to the drug in *erg6Δ* and *ERG6* cells (Fig. 2). Thus, Pdr5p exports cycloheximide efficiently, independent of *ERG6* function. As with dex, yeast lacking both *PDR5* and *ERG6* showed the highest sensitivity to cycloheximide, indicating that the effects of the two genes are additive.

The response to dex and the sensitivity to cycloheximide reflect indirectly the intracellular concentrations of the two drugs. To measure Pdr5p activity on the accumulation of a substrate directly, we determined the steady-state intracellular levels of the fluorescent drug rhodamine 6G. Exponentially

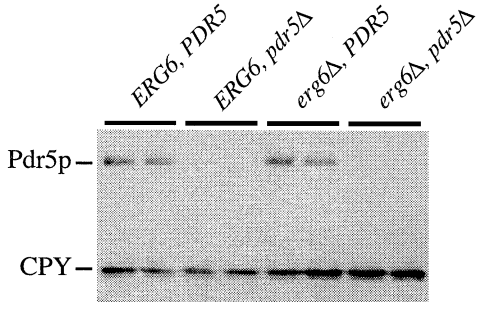

Fig. 5. *ERG6* and *erg6Δ* yeast express similar levels of Pdr5p. Pdr5p was detected by Western analysis of protein extracts from duplicate cultures of yeast strains YNK310 (*ERG6*,*PDR5*), YNK410 (*ERG6*,*pdr5Δ*), YNK589 (*erg6Δ*,*PDR5*) and YNK591 (*erg6Δ*,*pdr5Δ*), using an anti-Pdr5p specific antibody. An antibody against carboxypeptidase Y (CPY) was used to confirm comparable amounts of protein load for extracts from the different strains.

growing cells were incubated with rhodamine and analysed by flow cytometry. As shown in Fig. 3, the presence of Pdr5p decreased rhodamine accumulation by about 10-fold in both *ERG6* and *erg6Δ* cells, demonstrating that Pdr5p is active in *erg6Δ* yeast. As for the other substrates, *ERG6* by itself caused a significant decrease in the intracellular levels of rhodamine, and the effects of *ERG6* and *PDR5* were additive (Fig. 3).

To determine Pdr5p activity in a more direct manner, we measured the energy-dependent Pdr5p-mediated efflux of rhodamine 6G from intact cells. As seen in Fig. 4, Pdr5p led to a similar increase in the amount of rhodamine released into the medium from dye-pre-loaded *ERG6* and *erg6Δ* cells (164 and 194 fluorescence units/15 min, respectively, in the presence of glucose). To exclude the possibility that a reduced Pdr5p function could be compensated by higher expression levels in *erg6Δ* cells, we checked Pdr5p levels by Western analysis. Similar Pdr5p levels were seen in *ERG6* and *erg6Δ* cells, supporting the conclusion that *ERG6* does not affect Pdr5p activity (Fig. 5).

3.2. ERG6 determines the rate of passive drug diffusion

Our findings indicated that decreased drug efflux via Pdr5p could not explain the increased accumulation of small lipophilic drugs in *erg6Δ* cells. We asked next whether *ERG6* affects the passive diffusion of drugs across the plasma membrane. For this, we measured the rate of uptake of rhodamine 6G in cells that were depleted of energy. The energy-depleted state of the cells was confirmed by the lack of activity of the ATP-dependent Pdr5p transporter (Fig. 4 and data not shown). As seen in Fig. 6, rhodamine uptake was much faster in *erg6Δ* than *ERG6* energy-depleted cells, suggesting that *ERG6* function slows down the diffusion of lipophilic drugs across the membrane. Consistent with this, the rate of release of rhodamine 6G from de-energised intact cells was also higher in *erg6Δ* than *ERG6* cells (Fig. 4, compare rhodamine efflux in *erg6Δ* and *ERG6* cells in the absence of glucose.)

Changes in the rate of passive diffusion can affect the steady-state intracellular levels of drugs only if the drugs are actively maintained at a concentration gradient across the membrane. Accordingly, *ERG6* should have no effect on the steady-state rhodamine levels in energy-depleted cells. Indeed, flow cytometry measurements showed that de-energised *ERG6* and *erg6Δ* cells accumulated the same high steady-state levels of rhodamine 6G (data not shown), suggesting that lack of *ERG6* function and the ensuing increased diffusion rate simply enable drugs to attain a new equilibrium down their concentration gradient.

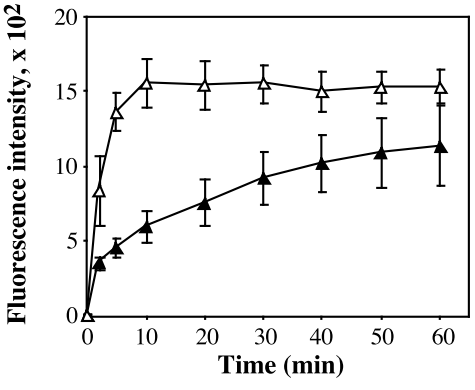

Fig. 6. Deletion of *ERG6* leads to an increased rate of passive diffusion for rhodamine 6G. Yeast strains YNK410 (*ERG6*,*pdr5Δ*) (▲) and YNK591 (*erg6Δ*,*pdr5Δ*) (△) were depleted of energy and incubated with 5 μM rhodamine 6G. Cell-associated fluorescence was measured in aliquots taken at the indicated times after addition of rhodamine, as described in Section 2. Data are the mean and range of results from duplicate cultures in five independent experiments.

4. Discussion

Several screens have identified *erg6* mutants as yeast with increased sensitivity to a broad range of chemical compounds [19,20,24]. This hypersensitivity of *erg6Δ* cells has proved useful in studies with small molecule inhibitors, such as Brefeldin A, that are ineffective in wild-type yeast [16–18]. Still, the mechanism by which *ERG6* mutations allow increased drug accumulation is not clear. At least two possibilities have been suggested. First, deletion of *ERG6* affects drug uptake directly, by rendering the membrane bilayer permeable to small molecules [14]. Second, deletion of *ERG6* acts indirectly, by compromising the activity of drug-efflux systems, such as Pdr5p [21]. In this study, we demonstrate that deletion of *ERG6* increases the rate of passive drug diffusion, thereby increasing drug uptake directly and not via Pdr5p.

Several of our observations support a direct effect of *ERG6* on drug resistance. First, the effects of *ERG6* and *PDR5* on accumulation of drugs are additive. Notably, an *erg6* mutant has been isolated due to its increased sensitivity to the steroid dex in a genetic screen that employed a *pdr5Δ* strain [24]. Second, Pdr5p activity is similar in *erg6Δ* and *ERG6* cells, as judged by its ability to confer resistance to dex and cycloheximide, lower rhodamine accumulation, and mediate rhodamine efflux. A previous study suggesting reduced Pdr5p activity in *erg6Δ* cells, has tested the activity of exogenously expressed Pdr5p rather than of the endogenous genomically expressed transporter; other *erg6Δ* defects, possibly in maintaining high copy number plasmids or overexpressing Pdr5p, may have led to the disparity in the results [21]. Finally, we show that the rate of rhodamine uptake is faster in *erg6Δ* than *ERG6* energy-depleted cells devoid of Pdr5p and other energy-dependent efflux activities. The faster passive diffusion of a drug across the membrane can lead to higher accumulation in *erg6Δ* cells, if the drug is subject to mechanisms that maintain a lower intra- than extra-cellular concentration. In other words, lack of Erg6p function facilitates diffusion down a concentration gradient. Interestingly, this implies that deletion of *ERG6* could lead to decreased intracellular accumulation of diffusible molecules that are actively concentrated inside the cell.

Erg6p methylates the side chain of sterols, a modification that is characteristic of membrane sterols of yeast, plants and parasites but not of higher eukaryotes. As a result, *erg6Δ* membranes contain predominantly zymosterol and cholesta-5,7,24-trienol rather than ergosterol [14,15,30]. Though the importance of sterols and lipids in determining membrane properties has been recognised for a long time, the degree to which ergosterol restricts the diffusion of small lipophilic molecules in yeast is still surprising. Other lipid modifying activities seem to have similar effects. Yeast lacking Pdr16p and Pdr17p have altered membrane sterol and lipid composition, display increased rates of passive drug diffusion, and are hypersensitive to many drugs [12]. Thus, plasma membrane lipid composition may be a useful target for novel antifungal drugs.

Compounds that inhibit Erg6p, or possibly Pdr16p and Pdr17p, could render pathogenic yeast more sensitive to already known and established drugs [2,31,32].

Multi-drug resistance in humans compromises the efficacy of chemotherapeutic regimens in the treatment of cancer. The increased expression of ABC transporters such as P-glycoprotein and MRP is a well established cause of resistance [1,3,7]. However, additional mechanisms may contribute. For example, plasma membrane ultrastructure can differ in multi-drug resistant, compared to sensitive cells [33]. Changes in the membrane sterol/phospholipid composition of resistant versus sensitive mammalian cell lines have been reported (reviewed in [10]). In many of these cases, it is still not clear whether or how these changes contribute to resistance. It will be important to establish whether membrane lipid composition contributes to multi-drug resistance in mammals, as in yeast, via mechanisms that are additive to the already known active efflux systems.

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