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Resistance and tolerance mechanisms to antifungal drugs in fungal pathogens

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Fungal pathogens are using several mechanisms to circumvent the inhibitory actions of antifungal drugs. The variety of these mechanisms was revealed in recent years by several laboratories with molecular approaches. Besides the identification of genes involved in antifungal drug resistance and the discovery of alternative pathways of resistance (i.e. biofilm formation), novel concepts in the understanding of the survival of fungal pathogen in the presence of antifungal drug (antifungal tolerance) have emerged.

Keywords: Fungi, antifungal agents, azoles, resistance, tolerance, multidrug transporters, cytochrome P450, biofilms, microarrays.

Introduction

Several classes of antifungal drugs are used today to treat fungal infections in humans. Depending on their antifungal properties, these drugs are used in specific clinical situations and for specific fungal species (see Table 1 for further details). The exposure of fungal pathogens to antifungal drugs has different consequences for their metabolism. First, cells can try to overcome the growth inhibitory action of antifungals by the development of various resistance mechanisms. These mechanisms will allow the growth of cells at a higher drug concentration than is the case for normal susceptible cells. Secondly, when the antifungal drug has reached a concentration resulting in growth inhibition, cells can mobilize factors determining if a given agent will be either fungistatic or fungicidal. This property is referred as to antifungal drug tolerance. This short review will lay emphasis on the current understanding of resistance and tolerance mechanisms to antifungal agents.

Established resistance mechanisms to antifungal agents

Resistance to antifungal drugs, which is understood as an increase in minimal inhibition concentration (MIC) as compared to values measured in reference susceptible organisms, has been reported in clinical situations for three classes of antifungal drugs: the polyenes, pyrimidine analogues (5-fluorocytosine) and the azoles (Sanglard & Odds, 2002). For other classes almost no cases have yet been documented until now. Table 1 summarizes the occurrence of resistance to several antifungal drugs among clinical isolates. The vast majority of clinical cases of antifungal resistance have been reported in their vast majority for the class of azoles. Acquisition of azole resistance in several fungal pathogens has probably been favoured by the repeated use of azoles (especially fluconazole) in treatments of HIV^+ patients with mucosal fungal infections in the period preceding the introduction of highly active antiretroviral therapy. Acquisition of azole resistance in fungal species has been observed mostly in *Candida* species including, with decreasing importance, *C. albicans*, *C. glabrata*, *C. dubliniensis*, *C. tropicalis* and, less frequently, in *Cryptococcus neoformans* in cases of meningitis (Kontoyiannis & Lewis, 2002). Azole resistance in systemic fungal infections of severely immuno-compromised patients is much less frequent and has been described mainly for *C. albicans* (Marr *et al*., 1997). Acquisition of azole resistance in filamentous fungi such as *Aspergillus fumigatus* causing life-threatening invasive diseases has also been observed in few cases in these patients after treatment with itraconazole (Warnock *et al*., 1999). Mechanisms of azole resistance have been the most extensively investigated in the recent years, since a high number of isolates has been available to research laboratories. These mechanisms involve:

– Alteration of the cytochrome P450 Erg11protein: Erg11p is responsible for the demethylation of

Table 1. Antifungal agents: activities against principal fungal pathogens, mode of actions and resistance mechanisms.

Antifungal	Spectrum/comments	Mode of action	Mechanism of resistance observed in clinical isolates
Polyenes - Amphotericin B	Broad activity against *Candida* spp., (except *C. lusitaniae*), *Cryptococcus neoformans* and filamentous fungi (except in the *Aspergillus* spp. *A. terreus* and *A. flavus*).	Binding to ergosterol and destabilization of cell membrane functions	Alteration in specific steps of ergosterol biosynthesis.
Pyrimidines analogues - 5-fluorocytosine (5-FC)	Active against *Candida* spp and *Cryptococcus* spp., however rapid emergence of resistance can appear when 5-FC is used as monotherapy.	Impairment of nucleic acid biosynthesis by formation of toxic fluorinated pyrimidine antimetabolites	- Decreased uptake of 5-FC. - Decreased formation of toxic antimetabolites.
Azoles - Fluconazole	Active against *Candida* spp. and *Cryptococcus* spp., less active against *C. glabrata* and no activity against *C. krusei*. No activity against filamentous fungi.	Inhibition of cytochrome P450 14α-lanosterol demethylase	- Enhanced efflux by upregulation of multidrug transporter genes. - Target alterations by occurrence of mutations. - Alteration of specific steps in the ergosterol biosynthetic pathway.
- Itraconazole	Like fluconazole, but enhanced activity against filamentous fungi.		
- Voriconazole	Like fluconazole, but enhanced activity against filamentous fungi, including *Aspergillus* and *Fusarium* spp.		
Allylamines - Terbinafine	Active against most dermatophytes, poor activity against *Candida* spp..	Inhibition of squalene epoxidase	Unknown
Morpholines - Amorolfine	Active against most dermatophytes, poor activity against *Candida* spp..	Inhibition of sterol Δ^{14} reductase and Δ^{7-8} isomerase	Unknown
Echinocandins - Caspofungin	Active against *Candida* spp. with fungicidal activity and moderately active against *Aspergillus* spp., poor activity against *Cryptococcus neoformans*.	Inhibition of the cell wall synthesis enzyme ß-1,3 glucan synthase	Unknown

lanosterol and is an important enzyme of ergosterol biosynthesis. Several mutations in *Erg11* alleles of azole-resistant *Candida* species have now been reported and are responsible for a decrease of affinity between azoles and the mutated Erg11p variants (Sanglard *et al*., 1998).

– Alteration of antifungal transport by enhanced efflux. In azole-resistant yeasts, genes encode two classes of drug efflux pumps in the cell membrane (ATP Binding Cassette (ABC)-transporters and Major Facilitators) are upregulated as compared to the corresponding most azole-susceptible species. Among ABC-transporters genes, *CDR1* (Candida Drug Resistance 1) and *CDR2* in *C. albicans* (Sanglard *et al*., 1997; Sanglard *et al*., 1995), *CdCDR1* in *C. dubliniensis* (Moran *et al*., 1998), a *CDR1* homologue in *C. tropicalis* (Barchiesi *et al*., 2000), *CgCDR1* and *CgCDR2* in *C. glabrata* (Sanglard *et al*., 2001; Sanglard *et al*., 1999) and *CnDR* in *C. neoformans* (Posteraro *et al*., 2003) have been identified as ABC-transporter genes up-regulated in azole-resistant isolates. Among Major Facilitators, *CaMDR1* (for Multidrug Resistance 1 and also previously known as *BEN*^{r} for Benomyl resistance) is up-regulated in some *C. albicans* azole-resistant yeast clinical isolates and several *CaMDR1*-like genes are also up-regulated in other *Candida* species such as *C. dubliniensis* (Wirsching *et al*., 2000; Wirsching *et al*., 2001) or *C. tropicalis C. tropicalis* (Barchiesi *et al*., 2000).

– Alterations in specific steps of the ergosterol biosynthesis pathway. Some steps of this pathway can be blocked by the occurrence of mutations of specific genes. The result of such alterations is the absence of synthesis of toxic sterol intermediate metabolites but also the absence of ergosterol. This type of alteration is very unfrequent in yeast clinical isolates.

In some studies investigating resistance mechanisms to azoles in clinical isolates, sequential isolates with stepwise increase in azole resistance, as measured by susceptibility testing, were available from patients treated with these compounds. Along with the molecular analysis of resistance mechanisms, it became clear that the stepwise increase could be explained by sequential acquisition of mechanisms. Several example have been reported documenting the multifactorial basis of azole resistance in clinical isolates (Perea *et al*., 2001; White, 1997a; White, 1997b).

Novel mechanisms of azole resistance

As mentioned above, azole resistance can be correlated with either the upregulation of ABC-transporter genes or Major Facilitator genes. In azole-resistant isolates however, other genes can be co-regulated with these transporters and might also be involved in azole resistance. Microarray experiments, with their ability to examine collections of genes differentially expressed in a genome, represent an attractive tool to identify clusters of genes co-regulated between azole-susceptible and azole-resistant isolates. The expression of co-regulated genes might be controlled by common regulatory circuits converging on the promoters of similar regulatory sequences in these genes. Consistent with this hypothesis, the upregulation of the ABC-transporter genes *CDR1* and *CDR2* is mediated by a common regulatory element (so called DRE, for Drug Responsive Element) situated in each promoter of these genes (De Micheli *et al*., 2002). In a study published by Cowen *et al*. (2002), the expression profiles of individual *C. albicans* isolates with reduced azole susceptibility were investigated, each of which was either upregulating *CDR1/CDR2* or *CaMDR1*. Interestingly, in an isolate upregulating *CDR2*, other upregulated genes were found and, under them, three (*YPL88 (LPG20), YOR49, YLR63*) contained in their promoter a consensus for a DRE, the presence of which is necessary for *CDR1* and *CDR2* upregulation. In the other isolates upregulating *CaMDR1*, several genes involved in oxidative stress response were also upregulated, thus suggesting that the oxidative stress pathway is contributing to *CaMDR1* upregulation. It is likely that in the future additional microarray experiments will be reported using other azole-resistant isolates as tester strains for expression profile analysis. These analyses will be helpful not only because they might cluster genes under the control of specific regulatory pathways, but also because they could reveal still unmasked azole resistance mechanisms.

Besides the main azole resistance mechanisms described above, alternative pathways for the acquisition of resistance can be used by fungi. An interesting alternative for developing azole resistance has been recently described. It uses the ability of fungal pathogens to build biofilms on synthetic or natural surfaces. Biofilms are organized as a dense network of differentiated cells onto which a layer of extracellular matrix can form. Biofilms can constitute a physical barrier for the efficient penetration of antifungals, which could explain that cells embeded in these structures can become recalcitrant to their activity. Measurement of drug susceptibilities in biofilms of *C. albicans* or *C. dubliniensis* yielded high MIC values for azoles and amphotericin B as compared to planktonic cells (Chandra *et al*., 2001; Ramage *et al*., 2001). As reported in *C. albicans*, the expression of genes involved in azole resistance (i.e. multidrug transporter genes) can also be altered in biofilms and may participate in the relatively high azole resistance measured in the cell population of these dense structures (Ramage *et al*., 2002).

Mechanisms of antifungal tolerance

Some important antifungal agents in use (fluconazole, itraconazole) have a fungistatic activity against *C. albicans*, while other compounds have strong (eg. amphotericin B) or moderate (eg. caspofungin) fungicidal activities in *C. albicans*. The fungistatic nature of azoles limits the efficacy of these substances especially in patients with deep immuno-suppression, since the immune system participates actively with azoles in the elimination of *C. albicans* from infected sites. Recently, a screening of different types of drugs encountered in medical practice able to potentiate the activity of azoles was undertaken. Among these different drugs, cyclosporin A was found to act in synergism with fluconazole and converted *in* its *vitro* fungistatic antifungal effect into a potent fungicidal drug (Marchetti *et al*., 2000b). This conversion into a fungicidal agent is due to a loss of tolerance. Tolerance to a given drug is generally defined as the capacity of an organism to survive in the presence of a drug at growth-inhibiting concentrations. The impact of the loss of fluconazole tolerance on drug efficacy has been tested in a rat model of endocarditis due to *C. albicans* infection (Marchetti *et al*., 2000a). The results of animal experiments confirmed those observed *in vitro*. The combination of fluconazole with cyclosporin A greatly increased the efficacy of fluconazole treatment and reduced fungal load to undetectable levels in infected organs. The molecular mechanisms of cyclosporin A on fluconazole tolerance in *C. albicans* is thought to be exerted at the level of the cyclophilin-cyclosporin A complex, which inhibits calcineurin functions. (See also Fig 1) Calcineurin is a

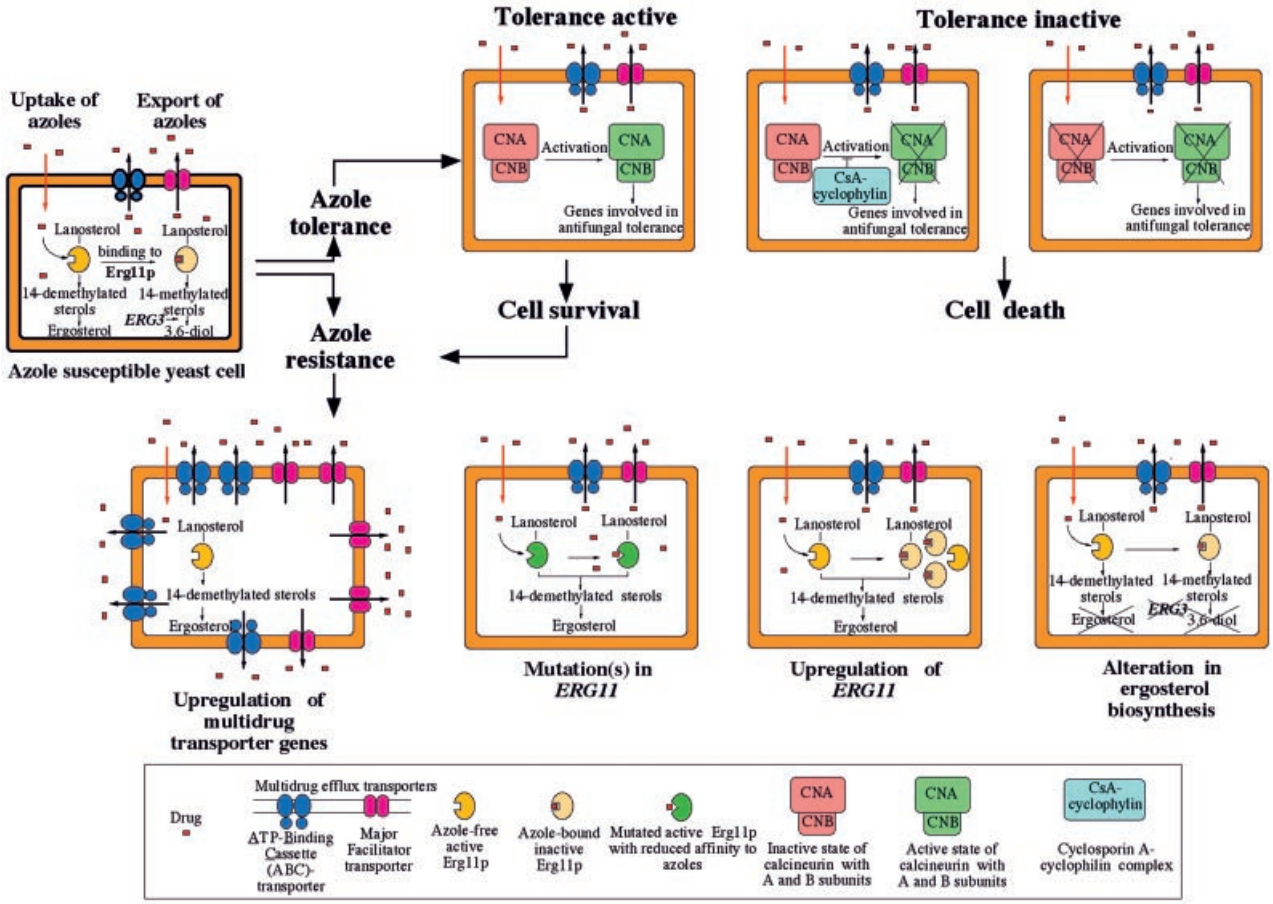

Fig 1 Resistance and tolerance mechanisms to azole antifungals. Azole-susceptible cells can be inhibited by low doses of azole antifungals after their uptake into the cells. The survival of C. albicans in the presence of azoles (tolerance) is dependent on the activation of calcineurin Inactivation of tolerance either by inhibition of calcineurin by the cyclosporin A-cyclophilin complex or by disruption of the genes encoding the calcineurin A (CNA) or B (CNA) subunits results in cell death in the presence of azoles. When tolerance to azoles exists, the development of azole resistance mechanisms (see text for details) indicated in the Figure can occur

protein phosphatase conserved among eukaryotes which is activated by a calcium-dependent pathway (Rusnak & Mertz, 2000). Recent studies report that calcineurin is essential for survival of *C. albicans* exposed to different environmental stresses and among them, exposure to antifungal agents is of relevance for medical mycologists (Cruz *et al*., 2002; Sanglard *et al*., 2003). The exact mechanisms by which calcineurin protect *C. albicans* from being killed in the presence of antifungal drugs remain to be elucidated. Moreover, calcineurin has several other functions in the maintenance of cell wall integrity and plays a critical role in the virulence of *C. albicans* (Sanglard *et al*., 2003). Calcineurin is also involved in the survival of other fungal pathogens (i.e. *C. krusei* or *C. glabrata*) in the presence of antifungal drugs (Bonilla *et al*., 2002; Cruz *et al*., 2002). Inhibition of calcineurin could therefore be utilized in the future for increasing the efficacy of specific antifungal drugs not only by interfering in their tolerance but also in the virulence of fungal species.

Conclusions

Studies on resistance and tolerance mechanisms to antifungal drugs have delivered the different resources utilized by simple microorganisms to circumvent the effect of growth inhibitory substances. Several basic biological processes which emerged from these studies will continue to be investigated and can be used for the purpose of new antifungal drug screening. Alternative pathways of antifungal resistance as observed in biofilm formation deserve research development. Genome-wide expression profiling will give the unique opportunity to obtain a comprehensive analysis of changes in gene expression in several clinical yeast isolates acquiring antifungal drug resistance over time, which until now, has been limited to a restricted number of genes and thus led to the description of a limited number of resistance mechanisms.

References

Barchiesi, F., Calabrese, D., Sanglard, D., Falconi Di Francesco, L., Caselli, F., Giannini, D., Giacometti, A., Gavaudan, S. & Scalise, G. (2000). Experimental induction of fluconazole resistance in *Candida tropicalis* ATCC 750. *Antimicrob Agents Chemother* **44**: 1578-84.

Bonilla, M., Nastase, K. K. & Cunningham, K. W. (2002). Essential role of calcineurin in response to endoplasmic reticulum stress. *Embo J* **21**: 2343-53.

Chandra, J., Kuhn, D. M., Mukherjee, P. K., Hoyer, L. L., McCormick, T. & Ghannoum, M. A. (2001). Biofilm formation by the fungal pathogen *Candida albicans*: development, architecture, and drug resistance. *J Bacteriol* **183**: 5385-94.

Cowen, L. E., Nantel, A., Whiteway, M. S., Thomas, D. Y., Tessier, D. C., Kohn, L. M. & Anderson, J. B. (2002). Population genomics of drug resistance in *Candida albicans*. *Proc Natl Acad Sci U S A* **99**: 9284-9.

Cruz, M. C., Goldstein, A. L., Blankenship, J. R., Del Poeta, M., Davis, D., Cardenas, M. E., Perfect, J. R., McCusker, J. H. & Heitman, J. (2002). Calcineurin is essential for survival during membrane stress in *Candida albicans*. *Embo J* **21**: 546-559.

De Micheli, M., Bille, J., Schueller, C. & Sanglard, D. (2002). A common drug-responsive element mediates the upregulation of the *Candida albicans* ABC transporters CDR1 and CDR2, two genes involved in antifungal drug resistance. *Mol Microbiol* **43**: 1197-214.

Kontoyiannis, D. P. & Lewis, R. E. (2002). Antifungal drug resistance of pathogenic fungi. *Lancet* **359**: 1135-44.

Marchetti, O., Entenza, J. M., Sanglard, D., Bille, J., Glauser, M. P. & Moreillon, P. (2000a). Fluconazole plus cyclosporine: a fungicidal combination effective against experimental endocarditis due to *Candida albicans*. *Antimicrob Agents Chemother* **44**: 2932-8.

Marchetti, O., Moreillon, P., Glauser, M. P., Bille, J. & Sanglard, D. (2000b). Potent synergism of the combination of fluconazole and cyclosporine in *Candida albicans*. *Antimicrob Agents Chemother* **44**: 2373-81.

Marr, K. A., White, T. C., van Burik, J. A. & Bowden, R. A. (1997). Development of fluconazole resistance in Candida albicans causing disseminated infection in a patient undergoing marrow transplantation. *Clin Infect Dis* **25**: 908-10.

Moran, G. P., Sanglard, D., Donnelly, S. M., Shanley, D. B., Sullivan, D. J. & Coleman, D. C. (1998). Identification and expression of multidrug transporters responsible for fluconazole resistance in *Candida dubliniensis*. *Antimicrob Agents Chemother* **42**: 1819-30.

Perea, S., Lopez-Ribot, J. L., Kirkpatrick, W. R., McAtee, R. K., Santillan, R. A., Martinez, M., Calabrese, D., Sanglard, D. & Patterson, T. F. (2001). Prevalence of molecular mechanisms of resistance to azole antifungal agents in *Candida albicans* strains displaying high-level fluconazole resistance isolated from human immunodeficiency virus-infected patients. *Antimicrob Agents Chemother* **45**: 2676-84.

Posteraro, B., Sanguinetti, M., Sanglard, D., La Sorda, M., Boccia, S., Romano, L., Morace, G. & Giovanni, F. (2003). Identification and characterization of a *Cryptococcus neoformans* ATP Binding Cassette (ABC) Transporter–encoding gene, CnDR1, involved in the resistance to fluconazole. *Mol Microbiol* **47**: 357-371.

Ramage, G., Bachmann, S., Patterson, T. F., Wickes, B. L. & Lopez-Ribot, J. L. (2002). Investigation of multidrug efflux pumps in relation to fluconazole resistance in Candida albicans biofilms. *J Antimicrob Chemother* **49**: 973-80.

Ramage, G., Vande Walle, K., Wickes, B. L. & Lopez-Ribot, J. L. (2001). Biofilm formation by *Candida dubliniensis*. *J Clin Microbiol* **39**: 3234-40.

Rusnak, F. & Mertz, P. (2000). Calcineurin: form and function. *Physiol Rev* **80**: 1483-521.

Sanglard, D., Ischer, F. & Bille, J. (2001). Role of ATP-binding-cassette transporter genes in high-frequency acquisition of resistance to azole antifungals in *Candida glabrata*. Antimicrob Agents Chemother 45, 1174-83.

Sanglard, D., Ischer, F., Calabrese, D., Majcherczyk, P. A. & Bille, J. (1999). The ATP binding cassette transporter gene CgCDR1 from *Candida glabrata* is involved in the resistance of clinical isolates to azole antifungal agents. *Antimicrob Agents Chemother* **43**: 2753-65.

Sanglard, D., Ischer, F., Koymans, L. *and* Bille, J. (1998). Amino acid substitutions in the cytochrome P-450 lanosterol 14alpha-demethylase (CYP51A1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. *Antimicrob Agents Chemother* **42**: 241-53.

Sanglard, D., Ischer, F., Marchetti, O., Entenza, J. *and* Bille, J. (2003). Calcineurin A of *Candida albicans* is involved in antifungal tolerance, cell morphogenesis and virulence. *Mol Microbiol* **48**: 959-976.

Sanglard, D., Ischer, F., Monod, M. & Bille, J. (1997). Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. *Microbiology* **143** (Pt 2): 405-16.

Sanglard, D., Kuchler, K., Ischer, F., Pagani, J. L., Monod, M. & Bille, J. (1995). Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. *Antimicrob Agents Chemother* **39**: 2378-86.

Sanglard, D. & Odds, F. C. (2002). Resistance of *Candida species* to antifungal agents: molecular mechanisms and clinical consequences. *Lancet Infect Dis* **2**: 73-85.

Warnock, D. W., Arthington-Skaggs, B. A. & Li, R. K. (1999). Antifungal drug susceptibility testing and resistance in *Aspergillus*. *Drug Resist Updat* **2**: 326-334.

White, T. C. (1997a). Increased mRNA levels of *ERG16*, *CDR*, and *MDR1* correlate with increases in azole resistance in *Candida albicans* isolates from a patient infected with human immunodeficiency virus. *Antimicrobial Agents and Chemotherapy* **41**: 1482-1487.

White, T. C. (1997b). The presence of an R467K amino acid substitution and loss of allelic variation correlate with an azole-resistant lanosterol 14-alpha-demethylase in *Candida albicans*. *Antimicrob Agents Chemother* **41**: 1488-1494.

Wirsching, S., Michel, S. & Morschhauser, J. (2000). Targeted gene disruption in *Candida albicans* wild-type strains: the role of the MDR1 gene in fluconazole resistance of clinical *Candida albicans* isolates. *Mol Microbiol* **36**: 856-65.

Wirsching, S., Moran, G. P., Sullivan, D. J., Coleman, D. C. & Morschhauser, J. (2001). MDR1-mediated drug resistance in *Candida dubliniensis*. *Antimicrob Agents Chemother* **45**: 3416-21.