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# Molecular basis of resistance to azole antifungals

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**The increased incidence of invasive mycoses and the emerging problem of antifungal drug resistance has prompted investigations of the underlying molecular mechanisms, particularly for the azole compounds central to current therapy. The target site for the azoles is the *ERG11* gene product, the cytochrome P450 lanosterol 14α-demethylase, which is part of the ergosterol biosynthetic pathway. The resulting ergosterol depletion renders fungal cells vulnerable to further membrane damage. Development of azole resistance in fungi may occur through increased levels of the cellular target, upregulation of genes controlling drug efflux, alterations in sterol synthesis and decreased affinity of azoles for the cellular target. Here, we review the adaptative changes in fungi, in particular *Candida albicans*, in response to inhibitors of ergosterol biosynthesis. The molecular mechanisms of azole resistance might help in devising more effective antifungal therapies.**

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Mucosal and invasive opportunistic fungal infections have increased during the past two decades as a consequence of the rising number of immunocompromised hosts, such as HIV-infected individuals, transplant recipients and patients given immunosuppressive therapy or broad-spectrum antibiotics. Besides the most commonly isolated *Candida* and *Aspergillus* species, new emerging opportunistic fungi include *Mucor*, *Fusarium*, *Rhizomucor* and *Absidia* species. In addition, the spectrum of infective fungi includes *Saccharomyces cerevisiae*. Another factor that contributes to the severity of opportunistic infections is the development of resistance to antifungal agents. Indeed, molecular alterations often result in the development of drug-resistant *Candida albicans* and other fungi from an initially susceptible population [1]. The aim of this paper is to review the molecular determinants of antifungal resistance, the understanding of which could provide new perspectives for improved treatment of invasive fungal infections confronting high-risk patients. Current treatment of systemic mycoses is mainly based on the use of polyenes (e.g. amphotericin B) and azoles, such as triazoles (e.g. itraconazole and fluconazole) (Fig. 1). Ergosterol, the major component of fungal membrane, is the target of polyene antibiotics and the ergosterol biosynthesis pathway is the target of azole derivatives. Ergosterol contributes to a variety of cellular functions, including fluidity and integrity of the membrane and the proper function of membrane-bound enzymes such as proteins associated with nutrient transport and chitin synthesis. Ergosterol is also a major component of secretory vesicles in *S. cerevisiae*, and has an important role in mitochondrial respiration; indeed, mutants defective in ergosterol biosynthesis and yeasts treated with azole compounds are induced to a respiratory-deficient 'petite' status at a high frequency [2]. This review focuses on the various mechanisms of azole resistance in *C. albicans*.

## Polyenes

Polyenes target ergosterol in fungal membranes. They are fungicidal agents used in short treatment regimens due to associated toxicity, which may account for the low incidence of resistance encountered. Consistent with this mode of action, amphotericin B-resistant *Candida* strains often have a marked decrease in ergosterol content compared with amphotericin B-susceptible control isolates [1]. Resistance in clinical isolates of *Cryptococcus neoformans* was also associated with a mutation preventing ergosterol biosynthesis at the C8-isomerization step [3]. Resistance may also be associated with altered phospholipids or increased catalase activity with decreasing susceptibility to oxidative damage [4].

## Other drugs

Flucytosine, which inhibits cellular DNA and RNA synthesis, is mainly used in combination therapy as

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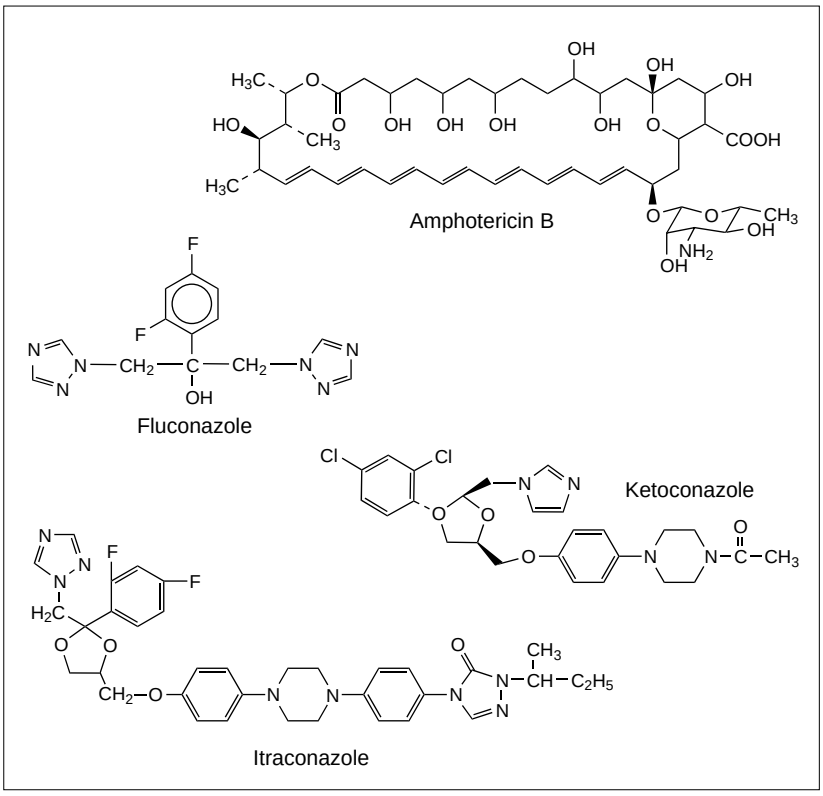


Fig. 1. Chemical structures of amphotericin B, ketoconazole, itraconazole and fluconazole. The imidazole group of ketoconazole and the triazole groups of itraconazole and fluconazole are responsible for the binding to the iron atom of the haem group of the cytochrome P450 C14α-lanosterol demethylase.

*Candida* was observed to become resistant at high frequency. Flucytosine resistance occurs in *C. albicans* strains bearing molecular variants of cytosine deaminase, uracil phosphoribosyl-transferase or uridine monophosphate-pyrophosphorylase [5].

Of the other antifungal drugs currently used, terbinafine and amorolfine also target ergosterol biosynthesis [1]. The former inhibits squalene epoxidase and the latter sterol C14-reductase and sterol $\Delta^{8-7}$-isomerase. Resistance may be detected with more extensive use in different clinical situations and can be expected also for other antifungals.

## Azole antifungals

Among the different categories of antifungal drugs, the development of azole resistance is the most relevant medical problem. Treatment failures have been observed following the extensive use of fluconazole for the management of *Candida* infections. In particular, resistance is associated with relapses of oropharyngeal candidiasis in AIDS patients, but azole resistance has also been observed in other settings and for different fungi. The first observations of azole resistance were in patients with chronic mucocutaneous candidiasis who were treated with ketoconazole (Fig. 1) [6]. Subsequent molecular investigations in these strains indicated that the same mechanisms were responsible for resistance in HIV-infected patients with fungal infections exposed to the next-generation drug fluconazole [7].

Azoles are directed against C14α-demethylase in the ergosterol pathway (Fig. 2). The resulting ergosterol depletion and accumulation of 14α-methyl-sterols (e.g. lanosterol and 14α-methyl-3-6-diol) interfere with the functions of ergosterol as the predominant cellular membrane component [8].

## Mechanisms of resistance to azoles

Extensive biochemical studies highlighted a significant diversity in the mechanisms conferring resistance to azoles. Since drug resistance can develop as a stepwise process over time, these mechanisms may combine with each other.

### *Reduced intracellular accumulation of azoles*

Intracellular accumulation of azoles can be reduced by the lack of drug penetration because of low ergosterol levels or possibly decreased ratio between phosphatidyl-choline and phosphatidyl-ethanolamine in the plasma membrane, which may change the membrane barrier function [9]. However, the vast majority of findings point to increased levels of active efflux of drugs being a prime mechanism of resistance. Most studies have focused on the amount of fluconazole in resistant cells and on the transcription levels of various transporters. Drug resistance is often associated with the upregulation of genes encoding efflux pumps. Increased mRNA levels of *Candida* drug resistance gene family (*CDR*), which are members of the ATP-binding cassette (ABC) transporter superfamily, and *MDR1*, a major facilitator with a much narrower substrate spectrum including fluconazole, have been associated with azole resistance. To date, at least seven *CDR* genes have been identified in *C. albicans* [10], but only *CDR1* and *CDR2* are so far associated with azole resistance [10–12]. There are indications that additional genes situated on chromosomes 3 or 4 might be associated with fluconazole resistance in *C. albicans* [13]. Monosomy of chromosome 4 occurring after 7 days of incubation with fluconazole and trisomy of chromosome 3, upon longer fluconazole exposure (35–40 days), may both produce fluconazole-resistant cells.

The role of the *CDR1* and *CDR2* transporters in transient azole efflux is supported by the loss of resistance to azoles and decrease in mRNA levels of the *CDR1* and *CDR2* genes after serial growth of resistant isolates in fluconazole-, itraconazole- and ketoconazole-free media [12]. A subsequent study also identified heterogeneous susceptibility to fluconazole among sub-populations of an individual isolate, an inducible transient resistance associated with changed gene expression of transporters [14]. This mechanism of azole resistance could be mediated by a change in cell wall components or the phenotypic switching of

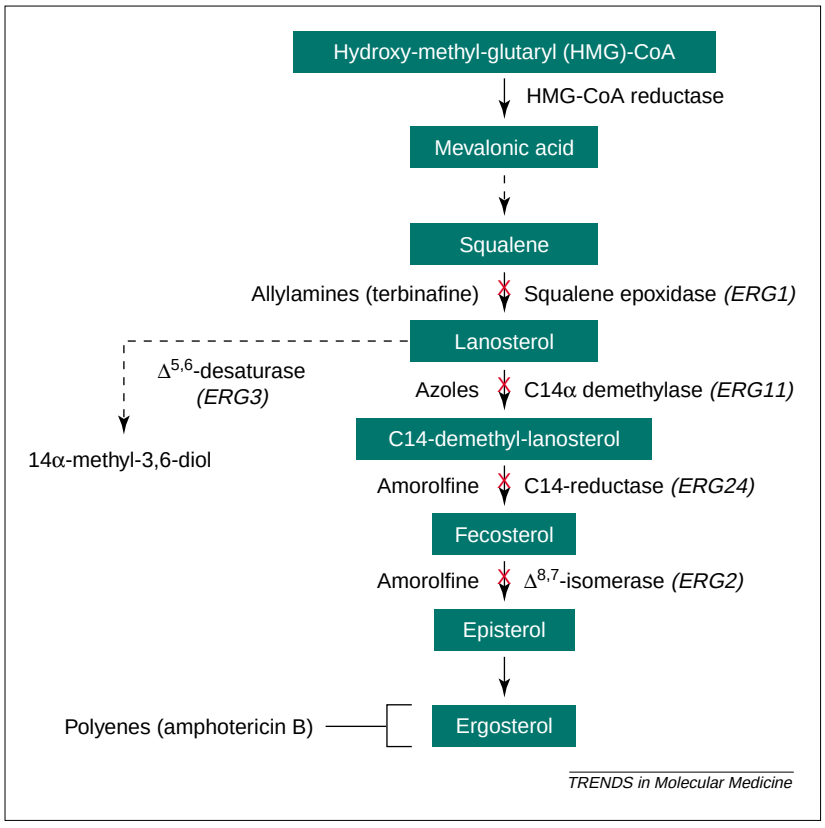


Fig. 2. Mechanism of action of antifungal drugs affecting the ergosterol biosynthetic pathway. The target enzymes are reported on the right with encoding genes in parentheses, whereas the antifungal drugs are reported on the left of the arrows indicating the sequential steps of sterol biosynthesis.

*C. albicans*. Indeed, Yoon *et al*. [15] recently observed that the development of amphotericin B-resistant *Candida lusitaniae* involves rapid *in vitro* switching from amphotericin-susceptible to amphotericin-resistant phenotypes.

Overexpression of *CDR1* and *CDR2* in *C. albicans* is associated with cross-resistance to the azoles, and amorolfine [16] and deletion of *CDR1* and *CDR2* confers hypersensitivity to azoles, amorolfine and terbinafine [17]; overexpression of *CDR1* and *ERG16* (commonly called *ERG11*) in fluconazole-resistant *C. albicans* correlated with increased cross-resistance to ketoconazole and itraconazole [18]. Although overexpression of genes encoding *CDR* increases the minimal inhibitory concentration (MIC) of BMS-207147, this agent can still be active against fluconazole-resistant *C. albicans* [19], in agreement with data published for *Candida dubliniensis* [20]. Increased mRNA levels can be the result of: (1) gene amplification; (2) increased transcription of the gene, which might depend on alteration of transcriptional activators which interact with the gene promoter or by mutations in the promoter itself; (3) increased half life of the mRNA, which might be related to slower degradation of the mRNA; and (4) changes in nuclear export, 5′-end capping, polyadenylation and RNA splicing. A recent study demonstrated that the increase in mRNA levels resulted from enhanced transcription of *CDR1*, *CDR2* and *MDR* [10].

Initially, most investigations were focused on fluconazole-resistant *C. albicans* strains. However, during the 1990s azole resistance in other *Candida* species, such as *Candida glabrata* and *Candida krusei*, has been observed [21], mostly in conjunction with the use of azoles. In these species intrinsic resistance or more rapid emergence can occur [22–24], and also increased efflux has been associated with resistance.

*C. glabrata* is becoming increasingly important since it is often recovered from immunocompromised patients suffering from oropharyngeal candidiasis [22,25] and candidemia [26,27]. *C. glabrata* is a haploid yeast, generally with intrinsic low susceptibility to azole derivatives, although it can acquire resistance to azoles upon exposure to these antifungals more frequently and rapidly than the diploid yeast *C. albicans*. This is presumably the result of both recessive and dominant resistance mechanisms. The transcription levels of the ABC transporters *C. glabrata CDR1* (*CgCDR1*) and, to a lower extent, *CgCDR2*, were increased in fluconazole-resistant *C. glabrata* strains [28]. However, when azole resistance was established in cells with a respiratory deficiency, petite mutants (rho-) lacking mitochondrial DNA, both *CgCDR1* and *CgCDR2* were upregulated [28]. Indeed, mitochondrial petite mutants have been associated with resistance elsewhere [29]. This suggests the existence of novel regulatory systems which enable the differential expression of these genes in *C. glabrata*. *C. glabrata* mitochondrial mutants might be clinically relevant as they have been isolated from stools of bone marrow transplant recipients undergoing fluconazole therapy [30]. The involvement of mitochondrial function in drug transporter regulation is intriguing, in particular in relation to the observation that azole treatment causes selection for petite status in *S. cerevisiae* [2]. The transcription of *PDR5*, an ABC transporter gene in *S. cerevisiae*, is induced in petite cells [31,32]. Accordingly, three different genes, all involved in mitochondrial function, *FZO1* [33], *OXA1* [34] and *MIM17* [35], act as negative regulators of *PDR5* expression. Further insight was provided by the observation that *S. cerevisiae* petite mutants lacking mitochondrial DNA induce nuclear genes coding for mitochondrial proteins (e.g. enzymes of the glycolytic pathway and of the citric acid cycle, cell wall components and also membrane transporters) [32]. All together these observations indicate that *PDR* gene expression is dependent on the mitochondrial function, suggesting that ABC transporter gene expression may be required for cell viability upon compromise of mitochondrial function. Therefore, further investigation is needed to understand the molecular basis controlling changes in expression of the transporters in fungi.

Other species of *Candida* have been encountered where a role for efflux explains intrinsic or acquired resistance. In line with previous observations,

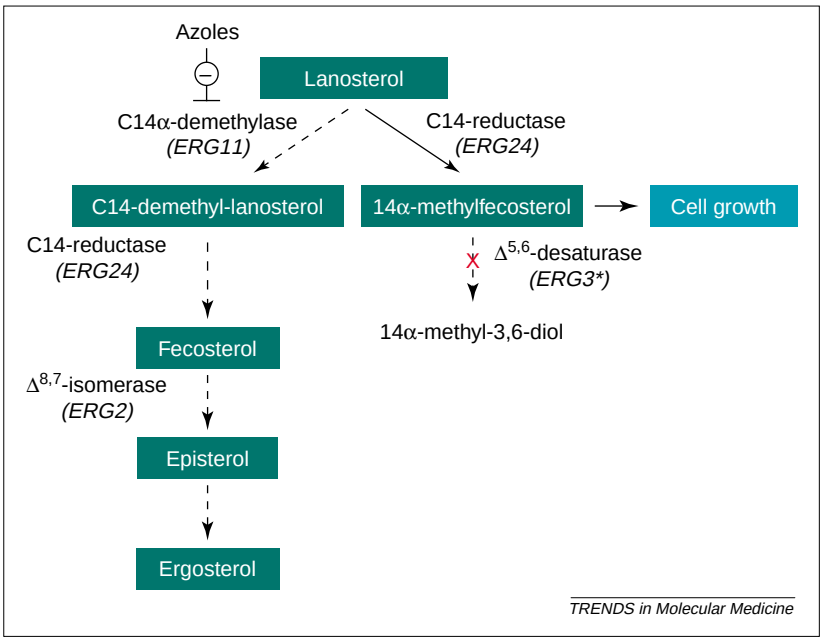


Fig. 3. Survival mechanisms in fungi during exposure to inhibitors of C14α-demethylase; *indicates mutation of *ERG3* gene; dotted line indicates reduced activity of the pathway, whereas solid line indicates preferential biochemical pathway.

*ABC1*, an ABC transporter, was overexpressed in miconazole-resistant *C. krusei* [24]. Similar to previous observations, development of fluconazole resistance in *C. tropicalis* in laboratory studies was correlated with increased transcription in the major facilitator, *MDR1*, and in ABC transporter *CDR1* with cross-resistance to itraconazole and terbinafine [36].

### *Increased levels of the azole cellular target*

The cellular target of azole antifungals is a cytochrome P450 that catalyzes sterol 14α-demethylation. This protein associated with the endoplasmic reticulum is encoded by *ERG11* (or *CYP51*) in yeast. Some studies looked at the potential of higher levels of the target protein or its altered regulation being a cause of resistance. In the case of a clinical isolate of *C. glabrata* cross-resistance to fluconazole, itraconazole and ketoconazole was caused by the duplication of the entire chromosome containing *CYP51*, thus inducing an increase in the copy number of *CYP51* gene as well as upregulation of 25 and downregulation of at least 76 genes which might elucidate underlying mechanisms of resistance [37]. However, the assessment of changes in protein level is fraught because of the multiplicity of CYPs in *Candida*. The transcription of the *ERG11* gene is altered following treatment of sensitive strains, presumably reflecting the response of the organism to depleted ergosterol or altered accumulated sterol. Within two hours of exposure of *C. albicans* to fluconazole or other azoles, including itraconazole, ketoconazole, clotrimazole, and miconazole, *ERG11* transcription is increased [38]. This response at the level of transcriptome is not an isolated event, since other genes of sterol biosynthesis undergo increased expression, such as *ERG9*, *ERG1*, *ERG7*, which encode enzymes that act upstream of *ERG11*, and *ERG25* and *ERG3*, which encode enzymes that act downstream of C14α-demethylase [38]. A recent complete transcription study confirms the complex upregulation of genes involved in ergosterol biosynthesis upon azole treatment by the use of microchip cDNA arrays [39], as observed previously for *S. cerevisiae* [40]. Further studies comparing sensitive and resistant strains will now be most valuable, as well as extension to comparisons at the protein level.

### *Alterations in sterol synthesis*

Some of the earliest genetic studies on azole resistance in *S. cerevisiae* pointed to alterations in sterol biosynthesis other than those caused by the *ERG11* gene product as a cause of resistance. Growth inhibition following azole treatment in *Candida* is caused by ergosterol depletion and its replacement by 14α-methyl-3,6-diol. This growth arrest can be circumvented if 14α-methylfecosterol accumulates instead. Mutants achieving resistance in this way have been observed on a number of occasions associated with AIDS patients, leukaemia and in the case of a patient suffering from chronic mucocutaneous candidiasis [11]. The changed sterol accumulation and resistance is achieved if cells are deficient in sterol $\Delta^{5,6}$-desaturase, encoded by *ERG3* [8] (Fig. 3). The altered sterol present during treatment allows continued growth and the production of functional membranes which are otherwise thought to be disrupted by the polar 6-OH group of the diol interfering with the sterol–phospholipid packing in the membrane. As a consequence of the *erg3* mutation the sterols of cells not treated with azole also do not contain ergosterol, and this absence of ergosterol provides a mechanism causing cross-resistance to antifungal amphotericin B [41].

Recent research is focusing on this mechanism, by using *S. cerevisiae*, the most genetically tractable yeast that is closely related to *C. albicans* and especially *C. glabrata*. The petite mutant (rho$^-$, lacking mitochondria) of this *S. cerevisiae* strain displayed resistance to fluconazole, and the mechanism of resistance of rho$^-$ mutants appears to involve uncoupling of oxidative phosphorylation. By contrast, petite derivatives of *erg3* were sensitive to fluconazole, and it has been suggested that in *erg3* mutants lacking mitochondria, a toxic sterol might accumulate under treatment [42]. As discussed above, previous studies on fluconazole sensitivity in petite strains have been made without seeing changes in fluconazole sensitivity [2]. Some differences in sterol accumulation patterns under treatment have been seen in a few resistant strains of *C. albicans* where obtusifolione accumulated, indicating interference in the process of 4-demethylation at the terminal

3-ketosteroid reductase step [43]. This may reflect a secondary mode of action. Ketosteroid products have been detected in several other sensitive fungi under azole treatment [1]. Other sterol gene alterations are not currently thought to cause resistance clinically, but the sterol composition of cells does affect drug sensitivity. Recent studies on the *S. cerevisiae ERG4* gene, which encodes the sterol C-24 reductase, examined the sensitivity of a strain on deletion of *ERG4*. The resulting accumulation of the precursor ergostan-5,7,22,24-tetraen-3β-ol correlated with hypersensitivity to several drugs, including miconazole, 4-nitroquinoline and fluconazole. The results were similar to effects seen in other sterol mutant strains [44]. These effects are presumably related to increased permeability of the cells with altered membranes, but a corresponding effect of increased resistance with increased ergosterol has not been identified.

### *Decreased affinity of azoles to cellular target*

Intrinsic resistance to fluconazole in *C. krusei* has been reported and the mechanism has been related to an insensitive target cytochrome P450 enzyme [45]. In addition, the presence of mutated *ERG11* was detected in fluconazole-resistant strains of *C. albicans* [46]. The location of the amino acid substitutions was considered in the light of a molecular model of the protein produced in the early 1990s in pioneering studies by Boscott and Grant [47]. One mutation, G464S, has emerged as a common resistance mutation in the haem-binding domain of the protein. As the drugs bind to the iron atom of the haem, the binding could be altered by changes in the position of the haem. Another mutation in the haem-binding domain, R467K, has been shown to cause resistance and was isolated from a patient treated with fluconazole [48]. Heterologous expression of the *C. albicans ERG11* gene in *S. cerevisiae* was used to study the effect of point mutations on resistance [49] and to distinguish which of the amino acid substitutions observed in clinical fluconazole-resistant strains conferred a primary effect altering resistance [50]. Further biochemical studies on heterologously expressed protein following site-directed mutagenesis for the substitutions G464S [51], Y132H [52] and R467K [53] revealed that the amino acid substitutions altered the affinity of the target molecule. The alterations G464S and R467K perturb the haem environment, causing a reduced affinity of fluconazole for cytochrome P450, but the protein retains catalytic activity, an essential prerequisite for the fungus. In the case of the R467K mutation, the specific activity of the mutated $CYP51^{R467K}$ is reduced four-fold as compared with fractions expressing wild-type *CYP51*. Furthermore, inhibition of activity of *CYP51* by fluconazole revealed a 7.5 fold-greater azole resistance of the variant protein than that of the wild type. These studies demonstrate that the clinically observed resistance was the result of the altered azole affinity to the fungal *CYP51* enzyme.

So far, the mutations Y132H, S405F, G464S, G465S, R467K and I471T have been established as causing resistance [49–56].

## Concluding remarks

Genome-wide transcription profiles, obtained by the use of microarrays, can be used to provide insight into the mode of action of an antifungal agent, as well as to characterize the response to perturbation of ergosterol metabolic pathway following exposure to a number of antifungal agents, including azoles and allylamines [39,40]. Indeed, the feasibility of such studies has recently been shown [57].

Finally, novel drug targets are being discovered by examination of the haploinsufficient phenotype, in which lowering the number of genes from two copies to one copy, results in a heterozygote that is sensitized to any drug that acts on the product of this gene [58]. By identifying the factors responsible for sensitivity to drugs and by screening patients before treatment, it will be possible to create an individualized chemosensitivity-chemoresistance map that may help in determining optimal individual treatment. In the future, the way it will be decided which drugs are to be given to each patient will include consideration of the pathogen as well as the patient. We are beginning to see how pharmacogenetics could represent a powerful tool to increase the likelihood of success of antifungal chemotherapy in seriously ill patients.

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