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Mechanisms of antifungal agent resistance

Yun-Liang Yang[1], Hsiu-Jung Lo[2]

[1]Department of Biological Science and Technology, National Chiao Tung University, Hsinchu and [2]Division of Clinical Research, National Health Research Institutes, Taipei, Taiwan, ROC

During the past decade, yeast infections have had an important role in nosocomial infections due to alterations in the immune status of patients. Coincidentally with the increased usage of antifungal agents, the number of reports of drug resistance has increased, which highlights the need for understanding the molecular mechanisms of antifungal agent resistance. This review describes the mechanisms of action of antifungal agents, cellular factors contributing to drug resistance, the known molecular mechanisms of drug resistance, and proposed but unproved molecular mechanisms of drug resistance. This review also proposes possible strategies for preventing drug resistance.

Key words: Antifungal agent, drug resistance, fungal infection, molecular mechanism

In the 1990s, there have been dramatic increases in the prevalence of fungal infections, which are probably the result of alterations in immune status associated with the acquired immunodeficiency syndrome (AIDS) epidemic, cancer chemotherapy, organ and bone marrow transplantation [1], and invasive hospital procedures. At the same time, drug resistance has become an important issue in a variety of fungal infections, which will have profound effects on human health. Oropharyngeal candidiasis due to drug-resistant fungi is a major problem for patients infected with human immunodeficiency virus (HIV) [2]. For instance, one third of late-stage AIDS patients had drug-resistant strains of *Candida albicans* in their oral cavities [3]. Approximately 11% to 40% of immunocompromised patients acquired systemic fungal infections and 30% of these infected patients died because of these infections [4]. In at least one major teaching hospital in Taiwan, *C. albicans* and other yeasts have become significant pathogens causing nosocomial infections in hospital-acquired infections since 1993 [5]. The rises in the prevalence of fungal infections and drug resistance have exacerbated the need for the next generation of antifungal agents.

Many currently available antifungal drugs have several problems including side effects, being ineffective against some fungi, and leading to the development of resistance. An increasing number of reports of clinical resistance to antifungal agents highlight the need for understanding the molecular mechanisms responsible for the development of drug resistance. *C. albicans* is the most frequently isolated fungal pathogen in humans and has caused morbidity in seriously debilitated and immunocompromised hosts. This review focuses on the pathogenic yeast *C. albicans,* in view of its potential clinical significance, and the available data for the mechanisms and factors accounting for its drug resistance.

The new liquid amphotericin B with improved safety profiles has been introduced [6,7] and new classes of drug, such as cancidas, candins, nikkomycins, and pradamicins-benanomicins are being studied [8-10]. However, among 15 different marketed drugs worldwide [9,10], the azole-based agents are currently the most widely used and intensively studied class of antifungal agent. Thus, this review further defines the focus on the molecular mechanisms of azole resistance and attempts to highlight research areas that need to be investigated in greater detail.

Mechanisms of Action of Antifungal Agents

A brief overview of the mechanisms of action of antifungal agents is provided here. Two recent reviews by White *et al* [1] and Ghannoum and Rice [11] have discussed this issue extensively.

Polyenes, ergosterol biosynthesis inhibitors, and 5-flucytosin (5-FC) are three common classes of antifungal agent (Table 1). The first two classes of drug act against ergosterol directly in some way. Ergosterol

Corresponding author: Dr. Hsiu-Jung Lo, Division of Clinical Research, National Health Research Institutes, 128, Section 2, Yen-Chiu-Yuan Road, Taipei, Taiwan, ROC.

Table 1. Properties of antifungal agents

Action target	Class	Type	Antifungal agent
Membrane (Enzymatic pathway)	Ergosterol biosynthesis inhibitors	Allylamines	Naftifine Terbinafine
		Thiocarbamates	Tolnaftate Tolciclate
		Azole-based	Ketoconazole Miconazole Fluconazole Itraconazole Voriconazole
Membrane (Composition)	Polyenes		Amphotericin B Nystatin
Protein synthesis	5-FC		5-FC

Abbreviation: 5-FC = 5-flucytosin

is the major sterol of the fungal plasma membrane that regulates the fluidity and asymmetry of membrane, and it is important for the proper functioning of many membrane-bound enzymes [12]. Polyenes, including amphotericin B and nystatin, target membranes containing ergosterol. Thus, they are effective only when organisms contain ergosterol in the plasma membrane, such as yeasts and algae. From the 1950s, amphotericin B has been the "best drug" of therapy for severe systemic mycoses.

Ergosterol biosynthesis inhibitors include three groups of antifungal agent: allylamines, such as naftifine and terbinafine; thiocarbamates, such as tolnaftate and tolciclate; and azole-based, such as imidazoles (including ketoconazole and miconazole) and triazole (including fluconazole, itraconazole, and voriconazole). These drugs interact with enzymes involved in the synthesis of ergosterol from squalene. Both allylamines and thiocarbamates inhibit early steps of ergosterol biosynthesis. The target of allylamines and thiocarbamates is squalene epoxidase [13,14], encoded by the *ERG1* gene [15]. The major target of azole-based drugs is cytochrome P-450 enzyme (lanosterol 14-α–demethylase) [12], encoded by the *ERG11* gene [16].

5-FC has a mode of action entirely distinct from the other two classes of antifungal agent. Aided by a cytosine permease, 5-FC enters into cells and is converted to 5-fluorouracil (5-FU) by a cytosine deaminase in the cells. 5-FU disrupts the synthesis of DNA and protein after it is converted into 5-fluoro-2'-deoxyuridine-5'-monophospate (5-FdUMP) and 5-fluoro-2'-deoxyuridine-5'-triphosphate (5-FdUTP), which are then incorporated into DNA and ribonucleic acid (RNA), respectively.

Factors Contributing to Antifungal Agent Resistance

The population of severely immunosuppressed patients has increased significantly during the past decade because of improved life-sustaining technologies, aggressive anticancer therapy, and the AIDS epidemic. The prevalence of fungal infections in patients lacking intact host defenses increases the dependence on antifungal agents for prophylaxis and therapy. Numbers of reports of resistance to antifungal agents have increased coincidentally with the increased usage of drugs.

Antifungal resistance can be described in two categories: clinical resistance and *in vitro* resistance. Clinical resistance is defined as a therapeutic failure due to an insufficiency of drug in serum and/or in tissue, and/or noncompliance with a medication regimen. Factors contributing to clinical antifungal agent resistance can be divided into three classes: fungal, agent, and host factors.

Fungal factors include the species, the minimum inhibitory concentration (MIC), cell types, and population of the organisms. The cell type of an organism can be identified by morphology and serology. The MIC of an organism can be determined by the microbiological laboratory in a hospital according to the antifungal susceptibility testing standards published by the National Committee for Clinical Laboratory Standard (NCCLS) [17]. The MIC of a clinical isolate obtained from *in vitro* assay is useful to predict the outcome of the clinical treatment. However, it is important to emphasize that the clinical success or failure in response to the administration of a specific antifungal agent depends on more than just one number, the MIC. Other factors discussed in this section also have important roles in determining the therapeutic outcome.

Agent factors include the class (such as polyenes, ergosterol biosynthesis inhibitors, and 5-FC), type (such as fungistatic or fungicidal), dosage, and pharmacokinetics. Understanding the pharmacokinetics of drugs is important, as clinical outcomes are dependent on how well the agent can be absorbed, how well the agent can be distributed to the correct location, and how stable the agent is. On the other hand, because different isolates of the same species have different MICs, the dosage of an antifungal agent is also a factor affecting clinical outcome. The frequency of drug resistance also increases with higher dosage of drugs.

The immune status of the host is one of the major factors determining clinical outcome. The site of infection, severity of infection, presence of foreign

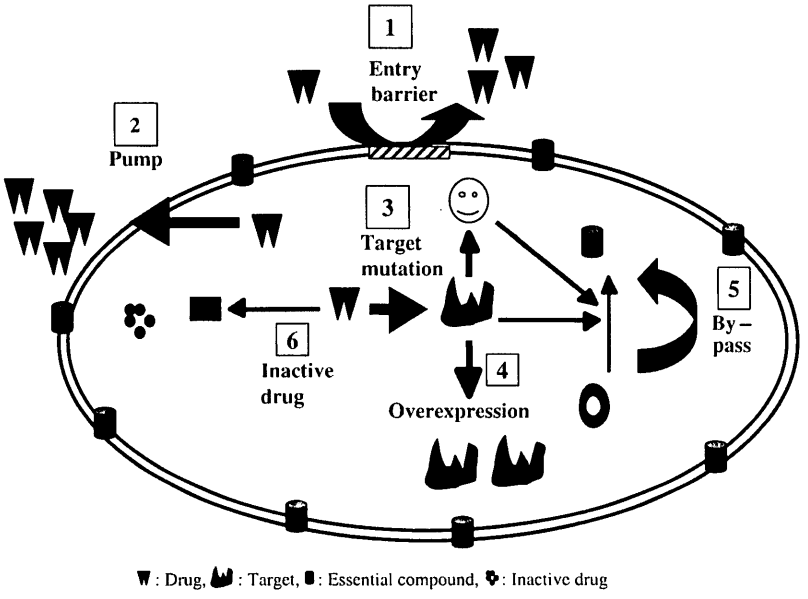

Fig. 1. Possible molecular mechanisms of antifungal agent resistance.
[1]Alterations of cell membrane and/or cell wall to prevent drug from entering.
[2]Overexpression of an efflux pump to reduce the accumulation of drug in the cell.
[3]Mutations in the drug target to prevent the drug from binding to the target.
[4]Overexpression of the drug target such that the drug fails to inhibit the biochemical reaction completely.
[5]Mutations in the other gene in the same pathway such that the cell bypasses the requirement for the product of the drug target gene.
[6]Inactivation or degradation of the drug.

materials, and compliance with the drug regimen are other host factors contributing to clinical resistance [1].

In vitro antifungal agent resistance can be described in two types: primary and secondary resistance. Primary resistance, also known as intrinsic or innate resistance, is defined as an organism's natural resistance to a drug prior to exposure. Secondary resistance, also known as acquired resistance, develops after exposure to a specific drug. The latter type of resistance has become frequently reported in immunodefective patients in the past decade.

For different fungal species, there are different levels of susceptibility to each antifungal drug. By antifungal susceptibility testing, the average MIC of a drug can be determined for each species. *Candida* species have various degrees of susceptibility to common antifungal agents. For example, *Candida krusei* and *Candida glabrata* are less susceptible to fluconazole than other *Candida* species [18-20], whereas *Candida lusitaniae* is relatively resistant to amphotericin B [21]. Historically, the majority of candidiasis was caused by *C. albicans*. The spontaneously mutated primary resistant species in a small number of the population have been considered as commensal organisms in healthy individuals. In the absence of drug, endogenous microbial populations would prevent those primary resistant species from becoming established. However, these primary resistant species may take over the microbiological population under selective pressure of antifungal agents. This may explain why the frequency of systemic fungal infections caused by non-albicans species has increased significantly [22,23]. The susceptible infecting organisms, in the presence of drugs, can become secondary resistant strains. The following section will discuss the potential molecular mechanisms of antifungal drug resistance. As the azole-based antifungal agents are currently the most widely used and extensively studied, we will focus on the azole resistance.

Mechanisms of Resistance

An antifungal agent has to enter into fungal cells, find its target, disrupt the cellular function of fungal cells, eliminate the infecting organisms, and establish a therapeutic success. There are many mechanisms contributing to a drug-resistant phenotype in infecting organisms (Fig. 1 and Table 2). The identified mechanisms

Table 2. Possible mechanisms of antifungal agent resistance

Mechanism	Alteration	Genes known to be involved	Reference
Reduction of the drug accumulation in cells	Mutation of membrane proteins		[20,35]
	Overexpression of efflux pumps	*CDR1* *CDR2*	[5,7,48]
		MDR1	[5,6]
Alteration of the target	Overexpression of the target	*ERG11*	[5]
	Point mutation of the target	*ERG11*	[48,49]
Alteration of other enzymes	Modification of drug Degradation of drug Bypass of target	*ERG3*	[39]
Alteration of chromosome	Copy number change		[46,47]

are: 1. reduction of drug accumulation, including preventing drugs from being imported into the cell and activating the efflux of drug from the cell; 2. alteration of drug target, including mutations of the target of the drug, overexpressing the target, and bypassing the drug-targeted enzyme by changing other enzymes in the same enzymatic pathway; and 3. inactivation of the drug, including modifying and degrading the drugs. A drug-resistant fungal cell has to employ at least one of these molecular mechanisms. This section discusses the resistant mechanisms of azole-based drugs, polyenes, and 5-FC; and other potential resistance mechanisms.

Azole-based drugs

Reduction of the drug import

The first-line mechanism for drug resistance is a defect in drug import. To prevent drugs from entering, cells can alter the composition of the membrane. Sterols and phospholipids are two major components of the cytoplasmic membrane. The interaction between these two regulates the fluidity and asymmetry [24] of membrane, which controls the transport of materials across the membrane. Mago and Khuller [25] and Hitchcock *et al* [26] also demonstrated that alteration in membrane composition contributed to a drug-resistant phenotype of *C. albicans*. However, direct correlation between the cytoplasmic composition and drug resistance has not been established. Little is known about how drugs enter a fungal cell. Hence, how fungal cells develop a mechanism to decrease the accumulation of drugs by preventing drug entry is still unclear.

Increase of efflux pumps

Orozco *et al* [18] determined the mechanism for the primary resistance of *C. krusei* to fluconazole. The cytoplasmic compositions of *C. albicans* and *C. krusei* have no significant differences. The capacity of fluconazole to enter cells is also similar in these two species. However, *C. krusei* can efflux fluconazole out of cells better than *C. albicans* can. A higher concentration of fluconazole is needed to inhibit the synthesis of ergosterol to the same extent in cell extracts from *C. krusei* than in those from *C. albicans*. This result suggested that in addition to efflux, the affinities of fluconazole to the target enzymes from these two species are also different.

At least two active efflux systems contribute to drug resistance: proteins belonging to adenosine triphosphate (ATP)-binding cassette (ABC) family and major facilitators (MF) family. Active efflux is one of the major mechanisms of resistance to azole-based antifungal agents and has been studied extensively.

The ABC transporters are proteins composed of four domains [27]. There are two integral membrane domains that span the membrane six or seven times and two ATP-binding domains that couple ATP hydrolysis to substrate transport [28]. The complete sequencing of the genome of *Saccharomyces.cerevisiae* has identified 33 ABC transporters grouped in several families. *PDR5 MRP/CFTR*, and *MDR* families are involved in drug resistance in a variety of organisms [1]. However, the *Candida* drug-resistance (*CDR*) genes in the ABC transporters are the only class of genes whose correlation with azole-based drug resistance has been established. The *CDR* genes were identified by complementing the hypersensitive phenotype to azole, cycloheximide, and chloramphenicol in the *pdr5* mutant in *S. cerevisiae* [29]. The levels of *CDR1* messenger RNA (mRNA) were higher in clinical azole resistance isolates than those in clinical azole susceptible ones [30,31]. A deletion of the *CDR1* gene results in hypersusceptibility to azole drugs as well as to terbinafine, amorolfine, and several metabolic inhibitors [32]. In contrast, susceptibility to

amphotericin B or 5-FC was not affected by mutation in the *CDR1* gene [33].

Like *CDR1*, *CDR2* was isolated for its complementation of a *pdr5* mutant of *S. cerevisiae*. Deletion or overexpression of *CDR2* does not have a strong effect on drug resistance [1]. Unlike the *cdr1/cdr1* mutant, the *cdr2/cdr2* mutant did not result in hypersusceptibility to azoles. However, the *cdr1/cdr1 cdr2/cdr2* double mutant was more hypersusceptible than the *cdr1/cdr1* single mutant [32]. The overexpression of *CDR2* was observed in the clinical resistant isolates from matched sets of susceptible and resistant ones [30]. More than eight other *CDR* genes have been identified by molecular and genetic manipulation [33]. However, correlation between these genes and drug resistance has not been established.

The other active efflux pumps, MFs, do not contain nucleotide-binding domains. They use the proton motive force of membranes as the source of energy. Antiport is the mechanism by which MFs transport antifungal drugs. Antiport means to pump substrate molecules out of the cell, whereas MFs have to pump protons into the cell. After the complete sequencing of the genome of *S. cerevisiae*, 28 MF genes have been identified [1]. However, *MDR1* is the only MF gene reported in *C. albicans* whose function correlates with drug resistance. *MDR1* was identified primarily for its ability to confer both benomyl and methotrexate resistance when transformed into *S. cerevisiae* [34]. This *MDR1* transformant is also resistant to cycloheximide, benztriazoles, and sulfometuron methyl [35].

Some clinical fluconazole-resistant isolates overexpress *MDR1* [30]. Overexpression of *CDR1* in *S. cerevisiae* causes resistance to azole-based antifungal agents. Unlike *CDR1*, strains with overexpression of *MDR1* are resistant to fluconazole but not to other azole-related drugs [31], suggesting that *MDR1* specifically transports fluconazole. The fact that a deletion of *MDR1* in *C. albicans* caused a hyper-susceptibility to fluconazole [36] is a direct genetic evidence that *MDR1* mediates fluconazole resistance in this pathogenic fungus.

Certain drug-resistant isolates have higher levels of active efflux pumps (*CDRs* and/or *MDR1*) mRNAs than susceptible strains do. The correlation between higher mRNA levels and drug resistance has been established [30,33]. The increase of mRNA level may be due to gene amplification, in which each copy of the gene is transcribed at normal level. Alternatively, the rate of transcription may be increased by either mutations in the promoter region or alterations of transcriptional factors. Levels of mRNA can also be increased by mutations that stablize the mRNA. Information regarding the mechanism of the increased mRNA level is limited.

Recently, the 5' flanking sequence of the *CDR1* gene has been characterized. The binding activity of the AP-1 site located in the promoter region of *CDR1* was increased by miconazole treatment [37]. According to the program of GCG Wisconsin Package (Version 10.1, Genetic Computer Group, Inc, USA), there are seven AP-1 sites located within 2000 base pairs of the 5' flanking sequence of the *MDR1* gene. If the AP-1 sites are the *cis*-regulatory elements for both *CDR1* and *MDR1* involved in drug resistance, identification of the factors interacting with these sequences should be addressed in the near future. Both *CDR2* and *MDR1* are expressed early during logarithmic growth and *CDR1* is expressed in all growth phases [38]. Understanding how the expression of *CDRs* and *MDR1* are regulated by *cis*-elements and *trans*-factors may be helpful to prevent drug resistance due to overexpression of the active efflux pumps.

Alteration of the target

One of the common mechanisms of drug resistance is the modification of the target enzyme and/or other enzymes in the same biochemical pathway. For azole-based antifungal drugs, it is the ergosterol biosynthetic pathway. The genes involved in the ergosterol biosynthetic pathway in *S. cerevisiae* (*ERG1, ERG2, ERG3, ERG4, ERG5, ERG6, ERG7, ERG11/ERG16, ERG24, ERG25, ERGX,* and *ERGY*) have been cloned [39-41]. To date, *ERG1* to *ERG12*, *ERG20*, *ERG24*, and *ERG25* have been identified in *C. albicans* [1,16,42]. There are three different mechanisms to develop a resistant phenotype through the ergosterol biosynthesis pathway: alteration of the target to prevent the drug from binding, increase in the expression of the target gene, and modification of other enzymes in the same pathway such that the cell can bypass the drug-targeted enzymes.

The predominant target enzyme of the azole-based drugs is lanosterol demethylase, a product of the *ERG11* gene. A point mutation in *ERG11* was identified from an azole-resistant clinical isolate. This point mutation results in the replacement of an arginine with lysine at amino acid 467 (R467K) of the *ERG11* gene [43]. Recently, more mutations such as G129A, Y132H, S405F, and G464S in the *ERG11* gene have been studied and are correlated with the occurrence of azole resistance in clinical isolates [44].

Knowledge about the regulation of *ERG11* expression is limited. The activity level of a gene can be raised by increasing the copy number of the gene,

the level of mRNA, and the efficiency of the protein. Gene amplification that results in an overall increase in expression is another common mechanism of drug resistance. Changes in the promoter sequence or alteration in transcriptional factors may also increase the rate of transcription. The amount of protein can also be increased by preventing the mRNA and/or protein from degradation. Recently, it has been reported that *ERG3* mutations are responsible for resistance by mean of bypassing the requirement of Erg11p for the synthesis of ergosterol [42].

Polyenes

Polyene resistance has not been a major clinical problem even though *Trichosporon beigelii*, *C. lusitaniae*, *Candida guillermondii*, *Pseudallescheria boydii*, and *Candida tropicalis* have been reported to be resistant to polyenes [45,46]. Alteration of the ratio of sterol to phospholipid [47-49], replacement of the polyene binding sterols by ones that bind polyenes less well (such as substitution of ergosterol by a 3'-hydroxy or 3-oxosterol [50]), and masking of existing ergosterol [51] are possible mechanisms contributing to polyenes resistance.

5-flucytosin

The three major mechanisms of 5-FC resistance are decrease in 5-FC uptake, prevention of 5-FC from being converted to 5-FU, and preventing 5-FU from converting to 5-FdUMP and 5-FdUTP. Usually, primary resistance to 5-FC is the result of a defect in cytosine deaminase, which prevents the drug from entering [2]. Secondary resistance to 5-FC results from a defect in uracil phosphoribosyl transferase, thus preventing 5-FU from converting to 5-FdUMP or 5-FdUTP [52]. Mutations in two genetic loci, FCY1 and FCY2, decreased the susceptibility to 5-FC [46]. The resistance to 5-FC is a common phenomenon in infectious microbes. Approximately 10% of *C. albicans* clinical isolates are intrinsically resistant, and 30% of isolates will develop secondary resistance [45]. Non-albicans and other non-*Candida* fungi have even higher rates of 5-FC resistance [53,54]. Thus, 5-FC is not used for treatment unless other antifungal agents have been administered.

Other potential mechanisms

Alteration of chromosome

Changes in the copy number of chromosomes by nondisjunction (loss of one copy of chromosome or gain of an extra copy of a chromosome) are beneficial for survival in different environments. *C. albicans* can grow in media containing sorbose by losing one copy of chromosome 5 [55]. The loss of one homologue of chromosome 4 or the gain of one copy of chromosome 3 was observed after incubation in media containing fluconazole [56]. One explanation for this event is that both *CDR1* and *CDR2* are located in the chromosome 3. Gain of one copy of chromosome 3 in the presence of fluconazole may help to efflux the drug out of cells due to an extra copy of *CDR1* and *CDR2*. Following the same line of reasoning, chromosome 4 may have some negative regulator(s) of efflux pumps. Thus, chromosomal nondisjunction may be a new mechanism of resistance to antifungal agents [56], even though no direct correlation has been established.

Cross-resistance

It is not surprising to find that clinical isolates that are resistant to azole are most likely cross-resistant to other azole drugs [1]. Overexpression of the ABC transporters such as *CDR1* and *CDR2* contributes to azole-based antifungal agent resistance, whereas overexpression of *MDR1* appears to be specific for fluconazole. However, the mechanisms causing the difference of these two efflux pumps have not been delineated. Which type of mutation in the *ERG11* can result in cross-resistance is not clear. Current data are insufficient to determine whether alteration in other enzymes in the ergosterol biosynthesis pathway contributes to cross-resistance. Alterations in the membrane structure or sterol context may result in either specific resistant phenotype for polyenes or cross-resistance to azoles [1,11,57].

Conclusion

Several points can be made about resistance to antifungal agents. There is no predominant mechanism contributing to the resistance of antifungal agents. One highly drug-resistant clinical isolate can be the result of a combination of two or more genetic alternations [30]. Alteration of the copy number of one of the chromosomes may suggest that gene amplification results in overexpression of active pumps or the target gene. Unlike resistance to antibiotics, antifungal drug resistance due to a plasmid-mediated mechanism has not been reported. Research to investigate the mechanisms of antifungal drug resistance is evolving. Some other potential mechanisms, such as preventing azoles or polyenes from entering the cell and degrading or modifying antifungal drugs in the cell, have not been reported.

One way to treat drug-resistant clinical isolates is to develop new agents. Voriconazole (UK-109 496),

Ravycibazike (ER-30346), D0870, Posaconazole (Sch56592), UR9646, and UR9751 are new azole-based antifungal agents that are being developed by several pharmaceutical companies (for a detailed review, see Sheehan *et al* [58]). To overcome the effects of antifungal agents, fungal cells might develop novel molecular mechanisms of drug resistance. Thus, with increased usage of different antifungal agents, we may expect an increasing number of clinical fungal pathogens become resistant to these agents.

A successful therapy can be defined as "a suitable agent prescribed to treat the right organism at appropriate dosage". Epidemiological studies by surveillance to determine the true frequency of antifungal resistance may be the first step to control the emergence of antifungal resistance. Rapid identification of fungal pathogens and the measurement of the MIC of clinical isolates *in vitro* may be helpful. Knowledge gained from studying the mechanisms of antifungal resistance may provide ideas on how to limit the emergence of resistance to those marketed antifungal agents and to develop safer and better compounds for the next generation of antifungal agents.

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