Review article

Mechanisms of azole resistance

Wenli Wang*
(Department of Pathogenic Fungi, Division of Phylogenetics)

Introduction

In the 1990s, drug resistance has become an important problem in a variety of infectious disease including human immunodeficiency virus (HIV) infection, tuberculosis, and other bacterial infections which have profound effects on human health. At the same time, there have been dramatic increases in the incidence of fungal infections, which are probably the results of alterations in immune status associated with the AIDS epidemic, cancer chemotherapy, and organ and bone marrow transplantation. The rise in the incidence of fungal infections has exacerbated the need for the next generation of antifungal agents, since many of the available drugs have undesirable side effects, are ineffective against new or reemerging fungi, or led to the rapid development of resistance. Although extremely rare 10 years ago, antifungal drug resistance is quickly becoming a major problem in certain populations, especially those infected with HIV (1). Oral candidiasis is one of the earliest and most frequent opportunistic infections associated with immune system failure in HIV-infected individuals. Candidiasis is usually treated effectively with oral fluconazole or other azoles. Recently, the efficacy of this drugs has been compromised by the emergence of azole-resistant strains of *Candida albicans* and intrinsically resistant species such as *Candida glabrata* and *Candida krusei*. One study estimated that $>$ 33% of isolates from patients with AIDS have MICs of fluconazole greater than 12.5 μg/ml, while standard susceptible strains have MICs of less than 4 μg/ml (2).

The AIDS epidemic, improved life-sustaining technologies, and aggressive anticancer therapy have contribute to today's severely immunosuppressed patient population who survive longer in the immunosuppressed state. Mucosal and systemic fungal infections are common in patients lacking intact host defences, increasing the dependence on antifungal agents for prophylaxis and treatment. Coincident with this increased usage, resistance has been observed (3). Recently, an elucidation of the molecular aspects of antifungal resistance has been initiated. Some clinical and cellular factors that contribute to antifungal drug resistance have been elucidated, and some molecular mechanisms have been described (table 1). The elucidation of the molecular mechanisms is still work in progress (3)

Mechanisms of action of azoles

Several inhibitors of the ergosterol biosynthetic pathway have been developed for use against medically important fungi, including allylamines, azoles, and morpholines. All of these drugs interact with enzymes involved in the synthesis of ergosterol from squalene, which is produced from acetate through acetyl coenzyme A, hydroxymethylglutaryl coenzyme A, and mevalonate. Ergosterol is an important sterol for fungi.

The azoles, including both imidazoles (ketoconazole and miconazole) and triazoles (fluconazole, itraconazole, and voriconazole), are directed against lanosterol demethylase in the ergosterol pathway. This enzyme is a cytochrome P-450 enzyme containing a heme moiety in its active site. The azoles act through an unhindered nitrogen, which binds to the iron atom of the heme, preventing the activation of oxygen which is necessary for the demethylation of lanosterol. In addition to the unhindered nitrogen, a second nitrogen in the azoles is thought to interact directly with the apoprotein of lanosterol demethylase. It is thought that the position of this second nitrogen in relation to the apoprotein

* Foreign researcher of the Research Center for Pathogenic Fungi and Microbial Toxicoses, Chiba University, Japan (1998-1999)
Address for correspondence: No. 8 Xishiku street, Department of Dermatology, the First Hospital of Beijing Medical University, Western District, Beijing 100034, China

may determine the specificity of different azole drugs for the enzyme. At high concentrations, the azoles may also interact directly with lipids in the membranes (3).

Mechanisms of azole resistance

Candida albicans is an imperfect, dimorphic yeast which is found as a commensal inhabitant of the human body. This organism is the most important and prevalent human fungal pathogen causing superficial as well as potentially life-threatening systemic mycoses. As the frequency of resistance has in creased in the HIV-infected population, the mechanisms that result in azole resistance have become a focus of attention.

It is important in the analysis of the mechanisms of resistance to use a matched set of isolates, that is, sensitive and resistant versions of the same strain, as determined by RFLP or karyotypig. This is required because *C. albicans* is mostly clonal; that is, grows vegetatively without sexual reproduction. The clonal nature of *C. albicans* makes it imperative that matched sets of sensitive and resistant versions of a singal strain be characterized when determining the mechanisms of resistance. RFLP analysis with the repetitive marker Ca 3 demonstrates a wide variety of RFLP patterns, suggesting that only rarely are clinical isolates related to each other (4). Specific mutations in a target gene can be the result of allelic variation or selection for a specific resistance phenotype. The differences can be determined only with a matched set of sensitive and resistant isolates from the same strain. To date, few studies have used matched sets of isolates (2, 5-10).

In 1994, Pfaller et al. (6) reported on an HIV-infected male who suffered from 14 episodes of oral candidiasis in 2 years who was treated with increasing doses of fluconazole. The MICs for a series of clinical isolates from the patient increased at intervals, starting with an MIC of 0.25 μg/ml and eventually rising to an MIC greater than 64 μg/ml. These increases correlated with the elevated doses of azole drug that were administered to the patient. Several techniques were used to demonstrate that the isolates from this series are the same strain of *C. albicans*, although it was shown that a substrain was selected at isolate 2. The resistance phenotype of the final isolate (isolate 17) is genetically stable, as it persists for over 600 generations in the absence of azoles. Recently, White (10) has shown that the overexpression of *MDR 1* (multidrug resistance), *ERG11* (Ergsterol biosynthetic enzymes), and *CDR* (*Candida* drug resistance) correlates with significant increases in resistance in the series. The series of 17 isolates collected during a 2-year period from a single AIDS patient has been studied extensively, because resistance to fluconazole developed gradually in this series and is correlated with the doses that were given to the patient (3).

(1) Cellular mechanisms of azole resistance

Several cellular factors can lead to the presence of a resistant strain in a patient: intrinsic resistance of endogenous strains, replacement with a more resistant species (*C. krusei*, *C. glabrata*), replacement with a more resistant strain of *C. albicans*, genetic alterations that render an endogenous strain resistant, transient gene expression that renders an endogenous strain temporarily resistant, alteration in cell type (yeast/haphae, switch phenotype), size and variability of the population, and population, bottlenecks, (3). In this review, alteration in cell type - one of cellular mechanisms of antifungal drug resistance will be discussed in detail.

C. albicans has a variety of different cell types which vary in their susceptibility to azoles. *C. albicans* can be divided into two serotypes, A and B, based on carbohydrate surface markers. The B serotype strains are more sensitive to azoles (especially ketoconazole) but are more resistant to 5-FC than are the A serotype strains (12).

C. albicans is a dimorphic fungus. The yeast-like or blastospore cell types are spherical, budding cells which are associated with commensal growth of *C. albicans*. Under a variety of conditions, *C. albicans* can form long, slender hyphal projections, which are often associated with *C. albicans* pathogenic. Azole drugs can interfere with hyphal production at 0.1 μM, 1/10 the therapeutic concentration of the drug *in vivo*. To date, there have been no studies monitoring the ability of resistant and sensitive clinical isolates of *C. albicans* to form hyphae. However, it is possible that a resistant strain with the ability to form hyphae even in the presence of azole drugs would be more pathogenic than a sensitive strain that is unable to form hyphae. This

possibility needs further study (3).

Very luckily, we obtained the above series of strains and other 4 widely studied azole-resistant isolates. Since hyphal formation has been implicated in the pathogenesis of *Candida albicnas* and azoles have been shown to inhibit hyphal formation, it was of interest to assess the hypha-forming capabilities of azole-sensitive *Candida albicans* and its resistant version (matched set) when antifungal agents are present. We thought having a higher capability in hyphal formation for azole-resistant *C. albicans* isolates than susceptible ones perhaps is one aspect of azole resistance mechanisms (cellular mechanisms). We compared the hyphal formation ability of this strains under different concentration of fluconazole (FCZ), itraconazole (ICZ), ketoconazole (KCZ), 5-flucytosine (5-FC) and amphotericin B (AmB). Our results clearly indicated that azole resistant *Candida albicans* strains had a significantly enhanced capability to form hyphae in the presence of different concentrations of azoles (13).

Our results and those in other reports (13, 14-18) indicated that cells which accumulate 14-α-methylsterols are seriously defective in hyphal formation. For example, a cytochrome p-450-deficient mutant of *Candida albicans*, strain D10, was employed to study the hyphal-forming capability. This strain was shown to be seriously defective in hyphal formation, but its wild-type reverent, strain D10R, was unaffected, suggesting that this function may be dependent on the 14-α-demethylation of lanosterol. Shimokawa (19) reported that among 75 polyene-resistant (presumably ergosterol) mutants of *Candida albicans*, only strain KD4700 accumulated 14-α-methylsterols and was defective in hyphal formation. This indicates that the presence of 14-α-methylsterols, rather than the absence of ergosterol, plays an important role in the inhibition of hyphal formation. Therefore the association of a specific membrane sterol composition with decreased growth rate and the inability to normally generate hyphae has important implications regarding the mechanism involved in hyphal formation as well as the mechanism of azole resistance. For an azole-resistant isolate, there is no 14-α-methylsterols accumulation in its membrane even in the presence of azoles, its capability of hyphal formation might be the same as that in the absence of azoles, so it can form hyphae and can cause infection even in the presence of azoles. And for the patient, his clinical manifestation is being infected with an azole-resistant isolate. Therefore, we can confidently say that the alteration in the capability of hyphal formation is also a kind of azole resistance mechanism, cellular mechanism. The substitution of 14-α-methylsterols for ergosterol results in a membrane with altered properties. Some authors reported that membrane permeability properties were altered and the sensitivity of the membrane to certain membrane-active agents was elevated. Hitchcock et al. (20) have reported that an azole-resistant demethylase mutant of *Candida albicans* was less permeable to azole. The authors concluded that this may be the basis of resistance. So the substitution of lanosterol for ergosterol in fungal membranes induces a variety of functional alterations such as permeability changes and leakage that result in growth inhibition or even drug resistance.

Table 1 : Potential molecular mechanisms of antifungal drug resistance

Alterations in drug import
Alterations in intracellular drug processing
Modification
Degradation
Alterations in the target enzyme
Point mutations
Overexpression
Gene amplification
Gene conversion or mitotic recombination
Alterations in other enzymes in the ergosterol biosynthetic pathway
Alterations in efflux pumps
ABC transporters
Major facilitators

(2) Molecular mechanisms of azole resistance

Resistance to antifungal drugs, specifically azoles such as fluconazole, in the opportunistic yeast *Candida albicans* has become an increasing problem in HIV-infected individuals. The molecular mechanisms responsible for this resistance have only recently become apparent and can include alterations in the target enzyme of the azole drugs (lanosterol 14-α-demethylase [14DM]), or in various efflux pumps from both the ABC transporter and major facilitator gene families (table 1) (3).

The accumulation of alterations has been documented for the series of 17 clinical isolates (2, 3, 10). Firstly, the *MDR1* efflux pump was overexpressed (10). Subsequently, three simultaneous changes occurred, all of which involved the *ERG11* gene: a point mutation, a gene conversion or mitotic recombination of the *ERG11* point mutation converting both alleles to the mutant version (2), and overexpression of *ERG11*(10). These changes resulted in an enzyme activity that was resistant to fluconazole. Finally, the *CDR* efflux pumps were overexpressed (10). Each of these alterations is likely to contribute in some way to the overall resistance phenotype of the final cell. At this time, there is no indication that the order of these alterations is important. It is likely that each would contribute to the resistance phenotype independent of the timing of the alteration. This gradual increase in resistance associated with specific alterations is an important mechanism of resistance.

Among the above molecular mechanisms which contribute to azole resistance, molecular alterations of the target enzyme encoding gene has been studied relatively thoroughly. As mentioned in the action mechanisms of azoles, the predominant target enzyme of the azole drugs is lanosterol demethylase. The gene encoding this protein is currently designated *ERG11* gene in all fungal species. Several genetic alterations have been identified that are associated with the *ERG11* gene of *C. albicans*, including point mutations in the coding region, overexpression of the gene, gene amplification (which leads to overexpression), and gene conversion or mitotic recombination.

In one study, a point mutation in *ERG11* was identified when an azole-resistant clinical isolate was compared with a sensitive isolate from a single strain of *C. albicans*. This point mutation result in the replacement of arginine with lysine at amino acid 467 of the *ERG11* gene (abbreviated R467K, where R=Arg and K=lys). The mutation is positioned near the cysteine which coordinates the fifth position of the iron atom in the heme cofactor. The mutation is thought to cause structural or functional alterations associated with the heme. Recent genetic manipulations suggest that R467K alone is sufficient to cause azole resistance. However, in the series of clinical isolates, several alterations occurred simultaneously (2), So it is impossible to determine how much R467K contributes to the overall azole resistance of the isolate.

Wang et al (21-25) selected thirty-two FCZ-resistant *C. albicans* as test strains (MICs $\geqq 64\ \mu g/ml$). With *ERG11* gene as object, and six sets of primers from the *ERG11* gene were chosen to amplify the interested fragments, and Southern blot hybridization, restriction fragment length polymorphism (RFLP) as well as single strand conformation polymorphism (SSCP) analyses were conducted for the fragments which were amplified by the six sets of primers (pre-resistant sensitive strains were used as controls). Three representative fragments, A66, D66 and E78, were selected to be cloned and sequenced. The PCR amplification showed that several tested strains were negative for some primers. However, Southern blot analysis reminded that their resistance did not result from the lacking of target enzyme coding gene. Single strand conformation polymorphism (SSCP) analysis showed that differences were noted between the resistant and sensitive strains and inter-resistant strains. Statistical analysis showed that the most variable sequence lied in the amplifier of the sixth pair of primer, and all the tested 32 strains showed positive results. In 11 mutation points they found, five resulted in amino acid alternation. It is likely that one or more mutational alterations (alone or in combination) might lead to the expression of an enzyme highly resistant to the inhibitory action of FCZ which in turn is responsible for the FCZ resistant trait in these strains.

How does (do) the point mutation (s) cause the resistance phenotype? Since 14DM (encoded by *ERG11* gene) is the target of azole antifungal agents, this enzyme is potentially prone to alterations leading to resistance to these agents. The activity of azole s is attributed to co-ordination of the azole moiety with 14DM haem. Azoles formed a one-to-one complex with the cytochrome (azole-14DM complex). Therefore, the efficacy of an azole as an inhibitor of 14DM depends on the geometric orientation of its azole moiety to haem iron. Clearly, it is possible that a decreased in the affinity of 14DM for these agents can cause azole resistance (25).

Nucleotide substitutions which resulted in amino acid changes may affect the geometric orientation of 14DM of those resistant strains, so that azoles can not interact with the haem iron or this inter-

action is interfered. Siglard et al. (26) analyzed in closer detail changes in the affinity of 14DM for azole antifungal agents. A research plan consisting of functional expression in *Saccharomyces cerevisiae* of the *Candida albicans ERG11* genes of sequential clinical isolates from patients was designed. This selection, which was coupled with testing of susceptibility to the azole derivatives fluconazole, ketoconazole, and itraconazole, enabled the detection of mutations in different cloned *ERG11* genes, whose products are potentially affected in their affinity for azole derivatives. This selection enabled the detection of five different mutations in the cloned *ERG11* genes which correlated with the occurrence of azole resistance in clinical isolates. These mutations were as follows: replacement of the glycine at position 129 with alanine (G129A), Y132H, S405F, G464S, and R467K. While the S405F mutation was found as a single amino acid substitution in a 14DM from an azole-resistant yeast, other mutations were found simultaneously in individual *ERG11* genes, i. e., R467K with G464S, S405F with Y132H, G129A with G464S, and R467K with G464S and Y132H. Site-directed mutagenesis of a wild-type *ERG11* gene was performed to estimate the effect of each of these mutations on resistance to azole derivatives. Each single mutation, with the exception of G129A, had a measurable effect on the affinity of the target enzyme for specific azole derivatives. So it was speculated that these specific mutations could combine with the effect of multidrug efflux transporters in the clinical isolates and contribute to different patterns and stepwise increases in resistance to azole derivatives (26).

In summary, in clinical isolates, it is unlikely that a single mutation will transform a susceptible strain into a highly resistant strain. Resistance usually arises after long periods in the presence of the drug, conditions that may support the gradual, stepwise development of resistance. It is more likely that resistance will gradually evolve over time as the result of several alterations due to continuous selective pressure from the drug. This suggests that several alterations will contribute to the final resistant phenotype (3).

References

1. Vanden Bossche, H., Warnock, D. W., Dupont, B., et al. : Mechanisms and clinical impact of antifungal drug resistance. J. Med. Vet. Mycol. 32 : 189-202, 1994.
2. White, T. C. : 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. Agent Chemother. 41 : 1488-1494, 1997.
3. White, T. C., Marr, K. A. and Bowden, R. A.: Clinical, cellular, and molecular factors that contribute to antifungal drug resistance. Clin. Microb. Rev. 11 : 382-402, 1998.
4. Schmid, J., Voss, E., and Ross, D. R. : Computer-assisted methods for assessing strain relatedness in *Candida albicans* by fingerprinting with the moderately repetitive sequence Ca3. J. Clin. Microbiol. 28 : 1236-1243, 1990.
5. Marr, K. A., White, T. C., Van, J. H., et al. : Development of a fluconazole-resistant *Candida albicans* in a patient undergoing bone marrow transplantation. Clin. Infect. Dis. 25 : 908-910, 1997.
6. Pfaller, M. A., Rhine, C. J., Redding, S.W., et al. : Variations in fluconazole susceptibility and electrophoretic karyotype among oral isolates of *Candida albicans* from patients with AIDS and oral candidiasis. J. Clin. Microbiol. 32 : 59-64, 1994.
7. Redding, S., Smith, J., Farinacci, G., et al. : Resistance of *Candida albicans* to fluconazole during treatment of oropharyngeal candidiasis in a patient with AIDS: documentation by in vitro susceptibility testing and DNA subtype analysis. Clin. Infect. Dis. 18 : 240-242, 1994.
8. Sanglard. D., Ischer, F., Monod M., et al. : Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob. Agent Chemother. 40 : 2300-2305, 1996.
9. Sanglard, D., Kuchler, K., Ischer, F., et al.: Mechanisms of resistance to azole antifungal agents in *Candida albicans* isolates from AIDS patients involve specific multidrug transporters. Antimicrob. Agent Chemother. 39 : 2378-2386, 1995.
10. White, T. C. : Increased mRNA levels of *ERG16*, *CDR*, and *MDR* 1 correlate with increases in azole resistance in *Candida albicans* isolates from an HIV-infected patient. Anti-

microb. Agent Chemother. 41 : 1482-1487, 1997.
11. White, T. C., Pfaller, M. A., Rinaldi, R. G., et al. : Stable azole drug resistance associated with a substrain of *Candida albicans* from an HIV-infected patient. Oral. Dis. 3 : S102-S109, 1997.
12. Vanden Bossche, H., Marichal, P., and Odds, F. C. : Molecular mechanisms of drug resistance in fungi. Trends Microbiol. 2 : 393-400, 1994.
13. Personal data.
14. Fekete-Forgacs, K., Szabo, E., and Lenkey, B. : The formation of hyphae of *Candida albicans* induced by cyclodextrins. Mycoses 40 : 451-453, 1997.
15. Belganger, P., Nast, C. C., Fratti, R., et al. : Voriconazole (UK-109, 496) inhibits the growth and alters the morphology of fluconazole-susceptible and-resistant *Candida* species. Antimicrob. Agent Chemother. 41 : 1840-1842, 1997.
16. Wakabayashi, H., Abe, S., Teraguchi, S., et al. : Inhibition of hyphal growth of azole-resistant strains of *Candida albicans* by triazole antifungal agents in the presence of lactoferrin-related compounds. Antimicrob. Agent Chemother. 42 : 1587-1591, 1998.
17. Ghannoum, M. A. : *Candida albicans* antifungal-resistant strains: studies on adherence and other pathogenicity related characteristics. Mycoses 35 : 131-139, 1992.
18. Wellmer, A. and Bernhardt, H. : Adherence on buccal epithelial cells and germ tube formation in the continuous flow culture of clinical *Candida albicans* isolates. Mycoses 40 : 363-368, 1997.
19. Shimokawa. O., Kato, Y., and Nakayama, H. : Accumulation of 14-methyal sterols and defective hyphal growth in *Candida albicans*. J. Med. Vet. Mycol. 24 : 327-336, 1986.
20. Hitchcock, C. A., Barrett-Bee, K. J. and Russell, N. J. : The lipid composition and permeability to azole of an azole- and polyene-resistant mutant of *Candida albicans*. J. Med. Vet. Mycol. 25 : 29-37, 1987.
21. Wang, W. L., Zhang B. R., Wang X. H., et al. : Screening of fluconazole-resistant *Candida albicans* strains using physical and／or chemical means. Acta Microbiologia Sinica 3 : 76-79, 1996. (In Chinese)
22. Wang, W. L., Li, S. Y., Wang, D. L., et al. : Primary study of azole antifungal resistance mechanisms of pathogenic yeasts. Chinese Journal of Dermatology 30 (5) : 332-336, 1997. (In Chinese)
23. Wang, W. L, Wang, D. L., and Li, R. Y. : Progress in the study of azole resistance (Review). Overseas Medicine-Division of Dermatology 29 : 234-237, 1997. (In Chinese)
24. Wang, W. L., Li, R. Y., Wang, D. L., et al.: Association of point mutations in the *ERG16* gene with azole resistance. Chinese Journal of Dermatology and Venereology In press. (In Chinese)
25. Wang, W. L., Zhu, L. H., Li, R. Y., et, al. : Resistance mechanisms of *Candida albicans* to azole antifungal agents. Chinese Journal of Medicine, In press. (In English)
26. Sanglard, D., Ischer, F., Koymans, L., et al. : Amino acid substitutions in the cytochrome P-450 lanosterol 14 α - demethylase (CYP51A 1) from azole-resistant *Candida albicans* clinical isolates contribute to resistance to azole antifungal agents. Antimicrob. Agent Chemother. 42 : 241-253, 1998.