Mechanisms of Azole Resistance in Candida: A Narrative Review

Neethu Babu, Chitralekha Saikumar, Jomon Raphael

ABSTRACT

Candida species, opportunistic fungal pathogens, pose a significant threat to human health, and the emergence of drug-resistant Candida strains has become a major concern in clinical settings, limiting the efficacy of antifungal therapies. Azoles are a class of antifungal agents that play a crucial role in the management of fungal infections. They have several advantages which contribute to their widespread use, like their broad-spectrum activity, availability in systemic and topical formulations, good oral bioavailability, tolerability, target specificity, established efficacy, and low toxicity for long-term prophylaxis. Azole resistance in Candida species poses a significant therapeutic challenge in the management of fungal infections, particularly in immunocompromised individuals. One common mechanism of resistance involves alterations in the target enzyme, cytochrome P450 14-α-demethylase (CYP51), which is essential for ergosterol biosynthesis, a crucial component of fungal cell membranes. Mutations in the CYP51 gene can lead to structural changes in the enzyme, reducing its affinity for azoles and thus decreasing their inhibitory effect. Another mechanism involves overexpression of efflux pumps, such as ATP-binding cassette (ABC) transporters and major facilitator superfamily (MFS) transporters. These pumps actively remove azoles from fungal cells, preventing them from reaching effective concentrations and thereby reducing their antifungal activity. A better understanding of the mechanisms underlying drug resistance is crucial for developing effective strategies to overcome this growing problem. This review describes and summarises the mechanisms associated with azole resistance in Candida, exploring the genetic, biochemical, and cellular factors contributing to this phenomenon. Additionally, potential approaches to combat azole resistance are discussed, aiming to pave the way for the development of novel therapeutic interventions.

Key Words: ATP Binding Cassette Transporters, Azoles, Drug efflux pumps, Drug resistance, Ergosterol biosynthesis, Mutation

INTRODUCTION

Candida species are opportunistic fungal pathogens that cause a wide range of infections, ranging from superficial mucocutaneous infections to life-threatening systemic diseases. Azole antifungal drugs, including fluconazole, itraconazole, and voriconazole have been widely employed in the management of Candida infections because of their wide-ranging effectiveness against various pathogens and comparatively low potential for toxicity. The new azoles like luliconazole, isavuconazole, itraconazole, isodaconazole, albaconazole are also active against various fungal infections. The non albicans Candida species which is being increasingly reported from various nosocomial infections as well as from infections in immunocompromised individuals are more resistant to azoles than the Candida albicans strains. The level of resistance to fluconazole in infections caused by Candida species varies depending on the infecting strain as well as the type of infection. Candidemia with C.albicans, in particular, have the lowest occurrence of azole resistance, but with oropharyngeal candidiasis (OPC) resistance is greater. C. glabrata demonstrates the highest prevalence of resistance to azole drugs and inherently shows reduced susceptibility to antifungals in the azole class. There is a debate regarding the increased infection rate of C. krusei, as it is intrinsically resistant to fluconazole. The controversy revolves around whether this higher infection rate is associated with fluconazole prophylaxis or prior treatment. Azole resistance in Candida is a complex phenomenon driven by various genetic and cellular mechanisms. The genetic basis of resistance involves alterations in the target enzyme, lanosterol 14α-demethylase (encoded by ERG11), which is essential for ergosterol biosynthesis. Mutations in ERG11 can lead to amino acid substitutions that reduce the binding affinity of azoles, rendering the antifungal drugs less effic-
In addition, upregulation of drug efflux pumps, such as the ATP-binding cassette (ABC) transporters Cdr1p and Cdr2p, can actively pump out azole drugs from the fungal cell, preventing their accumulation and promoting resistance.\textsuperscript{12,13}

Beyond genetic changes, the modulation of cellular processes contributes to azole resistance. Alterations in the sterol composition, including increased production of sterol intermediates and reduced ergosterol content, have been observed in azole-resistant \textit{Candida} strains.\textsuperscript{14} These changes can compensate for the inhibition of lanosterol 14a-demethylase and maintain membrane integrity and function despite azole treatment.\textsuperscript{15}

Furthermore, the formation of biofilms by \textit{Candida} is closely associated with drug resistance. Biofilms are complex communities of cells encased in a protective extracellular matrix, enabling \textit{Candida} to withstand high drug concentrations and evade host immune responses. Within biofilms, \textit{Candida} cells exhibit altered gene expression patterns and metabolic adaptations, contributing to reduced susceptibility to azoles.\textsuperscript{16,17,18}

This review aims to provide a comprehensive overview of the mechanisms associated with azole resistance in \textit{Candida}. By unravelling the intricate interplay of genetic, biochemical, and cellular factors contributing to resistance, this review endeavours to facilitate the development of effective strategies to combat azole-resistant \textit{Candida} infections, improving patient outcomes and preserving the efficacy of antifungal therapies.

The Azole antifungal agents

The azole antifungal agents can be classified into subgroups based on their chemical structure. The main subgroups of azoles are Imidazoles and triazoles.\textsuperscript{19}

\textbf{Imidazoles:} These are a subgroup of azoles that contain an imidazole ring in its chemical structure. Examples of various imidazole antifungal agents include: Clotrimazole, Miconazole, Econazole and Ketoconazole (although it is classified as an imidazole, ketoconazole also has some characteristics of a triazole).\textsuperscript{20}

\textbf{Triazoles:} Triazoles are a subgroup of azoles that contain a triazole ring in their chemical structure. Some examples of triazoles are fluconazole, itraconazole, voriconazole, isavuconazole, and posaconazole. Among these, fluconazole is the most frequently used triazole. The second generation of triazoles, including voriconazole, posaconazole, and isavuconazole, have shown higher effectiveness against resistant pathogens. Isavuconazole, in particular, is a newly developed azole that is as effective as voriconazole but has a lower toxicity level.\textsuperscript{20}(Table 1)

\section*{Mechanism of action of the Azole antifungal agents}

Azoles exert their antifungal activity by targeting a specific enzyme called cytochrome P450 14-alpha-demethylase (CYP51). This enzyme is essential for the synthesis of ergosterol, a crucial component of the fungal cell membrane. By inhibiting CYP51, azoles disrupt the synthesis of ergosterol, leading to the accumulation of abnormal sterols and disruption of the fungal cell membrane’s integrity.\textsuperscript{21}

The mechanism of action of azoles involves binding to the heme group within the active site of CYP51. This binding prevents the enzyme from converting lanosterol, a precursor molecule, into ergosterol. Consequently, the fungal cell membrane becomes depleted of ergosterol and accumulates toxic sterol intermediates, such as 14-alpha-methylsterols.\textsuperscript{22}

The absence of sufficient ergosterol weakens the cell membrane’s structure and function, impairing its ability to regulate ion transport and maintain integrity. This disruption leads to increased membrane permeability, leakage of cellular contents, and ultimately, cell death.

Additionally, azoles may have effects on other enzymes involved in fungal metabolism, such as those responsible for the biosynthesis of other essential lipids or cell wall components. However, the primary mechanism of action for azoles is the inhibition of CYP51 and subsequent disruption of ergosterol synthesis, which is crucial for fungal cell membrane integrity.\textsuperscript{23}

\section*{Mechanisms of azole resistance}

\textbf{Mutations in ERG11 gene:} Azole resistance in fungi can occur through mutations in the ERG11 gene, which codes for the cytochrome P450 14-alpha-demethylase enzyme targeted by azole drugs. These mutations can lead to changes in the structure or activity of the enzyme, reducing the binding affinity of azoles and rendering them less effective.\textsuperscript{24} This occurs by mechanisms like

\begin{itemize}
  \item[a)] Alteration of the target enzyme: Mutations in the ERG11 gene can result in amino acid substitutions or deletions in the cytochrome P450 enzyme.\textsuperscript{25} These changes can affect the three-dimensional structure of the enzyme, particularly in the region where azoles bind. As a result, the mutated enzyme may have reduced binding affinity for azoles, making the drugs less effective in inhibiting the enzyme’s activity.
  \item[b)] Overexpression of the ERG11 gene: In some cases, azole resistance can be associated with an increase in the expression of the ERG11 gene.\textsuperscript{26} It may be due to 3 mechanisms-
\end{itemize}

Overexpression of the ERG11 gene leads to an elevated production of the cytochrome P450 14-alpha-demethylase enzyme. The higher levels of the enzyme can compensate for the inhibitory effects of azole drugs, as there is a larger
pool of the target enzyme available for the drug to bind and inhibit. This increased enzyme production ensures that sufficient functional enzyme is present, even in the presence of azole drugs. Sony Paul et al. conducted a research study on HIV patients and observed multiple nonsense and missense mutations. These mutations were found to be the cause of the enzymes losing their capability to bind with azoles.  

Fungal cells can develop mechanisms to actively pump out azole drugs, reducing their intracellular concentration and effectiveness. Mutations in ERG11 can upregulate the expression of efflux pump genes, such as those belonging to the ATP-binding cassette (ABC) transporter family. This process involves the creation of an isochromosome, where a chromosome arm containing genes for both a transcription factor that controls ABC transporters and the target of azoles, Erg11, is replicated. Such duplications of chromosomes result in an elevated number of genes encoding efflux pumps. These pumps can actively transport azoles out of the fungal cell, limiting their accumulation and preventing their therapeutic action.  

**Mutations in ERG3 gene:** The ERG3 gene encodes the enzyme sterol Δ(5,6)-desaturase, which is responsible for an essential step in the ergosterol biosynthesis pathway. Ergosterol is a vital component of the fungal cell membrane, similar to cholesterol in human cells. Azole antifungal drugs target the ergosterol biosynthesis pathway by inhibiting the enzyme cytochrome P450 14-alpha-demethylase (CYP51). However, mutations in the ERG3 gene can lead to azole resistance by altering the function or expression of the sterol Δ(5,6)-desaturase enzyme. These mutations can result in loss of function or decreased activity of the enzyme, affecting the conversion of the precursor molecule, obtusifoliol, into ergosterol. When the function of sterol Δ(5,6)-desaturase is impaired, the ergosterol biosynthesis pathway is disrupted. As a compensatory mechanism, the fungal cell may produce alternative sterols or sterol intermediates that can partially replace ergosterol in the cell membrane. These altered sterols have different structural properties and may not be as susceptible to the inhibitory effects of azole drugs. As a result, the reduced production of ergosterol, along with the presence of alternative sterols, contributes to decreased susceptibility to azole antifungals. The altered composition of the fungal cell membrane reduces the drugs’ effectiveness in disrupting membrane integrity and function, leading to resistance.  

**Mutations in ERG5 gene:** Mutations in the ERG5 gene can also contribute to azole resistance in fungi. The ERG5 gene encodes the enzyme C22-sterol desaturase, which plays a crucial role in the ergosterol biosynthesis pathway. The C22-sterol desaturase enzyme is responsible for introducing a double bond at the C22 position of sterol intermediates in the ergosterol biosynthesis pathway. This step is necessary for the conversion of the intermediate 24(28)-methylene dihydrolanosterol into zymosterol, a precursor of ergosterol. Mutations in the ERG5 gene can lead to amino acid substitutions or other alterations in the C22-sterol desaturase enzyme. These mutations can affect the enzyme’s structure or activity, resulting in reduced or impaired function. Consequently, the conversion of 24(28)-methylene dihydrolanosterol to zymosterol is compromised. As a compensatory response, fungal cells with ERG5 mutations may bypass the C22-sterol desaturase step by utilizing alternative pathways. These alternative pathways can involve the production of different sterols or sterol intermediates that can substitute for ergosterol in the cell membrane. The altered sterol composition in the cell membrane, along with the reduced production of ergosterol, contributes to azole resistance. According to a study conducted by Claire M Martel and colleagues on *C. albicans*, they have documented a strain that possesses mutations in both the ERG11 and ERG5 genes. These mutations provide a specific benefit by rendering clinical isolates resistant to treatment with Amphotericin B.  

**Alterations in drug transport mechanisms:** Alterations in drug transport mechanisms, specifically the overexpression of transporter genes, play a significant role in azole resistance in various fungal species. There are two classes of drug transporters involved: the major facilitator superfamily (MFS) transporters and the ATP-binding cassette (ABC) transporters (summarised in Table 2). The MFS transporters utilize the proton gradient across the plasma membrane for drug efflux, while the ABC transporters hydrolyse ATP to drive drug efflux. Studies on *Candida* species have been facilitated by investigating the pleiotropic drug resistance phenotype in *Saccharomyces cerevisiae*, primarily driven by ABC transporters like Pdr5p.  

**Over expression of ABC transporter genes**  
Candida Drug Resistance 1(CDR1) and Candida Drug Resistance 2(CDR2) are ABC transporter genes that encode efflux pumps located in the cell membrane of fungal cells. Overexpression of these genes results in an elevated production of Candida Drug Resistance Protein, Cdr1p and Cdr2p efflux pump proteins. The overexpression of CDR1 and CDR2 genes and subsequent increased production of Cdr1p and Cdr2p efflux pumps provide a mechanism for certain *Candida* species, particularly *C. albicans*, to actively pump out azole antifungal drugs from their cells. This efflux-mediated resistance mechanism reduces the intracellular drug concentration and limits the drugs’ effectiveness, making the treatment of *Candida* infections more challenging.  

Cdr1p and Cdr2p efflux pumps are known to have broad substrate specificity, which means they can pump out not only azole drugs but also other structurally related compounds. This ability allows the efflux pumps to confer resistance not only to azoles but also to other antifungal agents, further complicating the treatment of fungal infections.
CDR1 and CDR2 genes can be co-regulated, meaning their expression levels can be upregulated together. This co-regulation can lead to a synergistic effect, further enhancing the efflux pump activity and resistance toazole drugs.

The ABC transporters CDR1 and CDR2 were first identified in *C. albicans* and significantly contribute to azole resistance. Disruption of CDR1 results in hyper susceptibility to azoles, while overexpression of CDR1 or CDR2 increases fluconazole resistance. CDR1 is more influential than CDR2 in azole resistance. Homologs of CDR1 and CDR2 have been identified in *C. dubliniensis* and other *Candida* species, but their clinical significance may vary.

ABC transporters CDR1, PDH1, and SNQ2 play a major role in azole resistance in *Candida glabrata* clinical isolates. Disruption of CDR1 and PDH1 increases fluconazole susceptibility, while SNQ2 overexpression contributes to resistance. *C. glabrata* strains obtained from clinical samples exhibit a moderate natural resistance to azole drugs. Furthermore, it has been observed that this inherent resistance can intensify during the course of treatment or prophylaxis with azoles, particularly fluconazole. Even after the treatment ends, the resistance persists. It is important to note that the widespread use of fluconazole has led to an increase in cross-resistance in *C. glabrata* against other azoles such as itraconazole, ketoconazole, and voriconazole. Researchers have identified around 18 ABC transporters in *C. glabrata*, among which CgCDR1, CgCDR2, and CgSNQ2 function similarly to CDR1 and CDR2. Overexpression of CgCDR1 has been found to confer resistance to azoles.

CDR1 appears to be more significant in azole resistance in *C. tropicalis* and *C. krusei* strains also, but their roles in clinical isolates are yet to be established.

**Over expression of MFS transporter genes**

The first transporter implicated in antifungal resistance was an MFS efflux pump from *C. albicans*, initially called BENr. This transporter, later renamed multidrug resistance 1 (MDR1), confers resistance to various compounds, including azoles like fluconazole and voriconazole. MDR1 expression is induced upon exposure to certain compounds and has been found to be upregulated in many fluconazole-resistant clinical isolates. Disruption of MDR1 reduces resistance, while overexpression increases resistance tofluconazole. Azole resistance mechanism associated with membrane transporters has been observed in *C. auris*, a fungal pathogen that is gaining recognition as a multidrug-resistant threat.

In a study on *C. auris* by Jizhou Li et al. it is demonstrated that MDR1 is responsible for reduced azole susceptibility. Among the entire set of MFS proteins, only CaMdr1p is recognized for its ability to expel drugs, and an increase in its expression has been associated with the development of azole resistance.

MFS transporters have also been identified in other *Candida* species, such as *C. dubliniensis*, *C. glabrata*, and *C. tropicalis*. These homologs, like CdMDR1 and CgFLR1, have been associated with fluconazole resistance in laboratory strains but show variability in clinical isolates. Costa et al. in their study findings suggest that there is only a minor role for MFS transporters in the drug resistance of *C. glabrata*. Another MFS transporter influencing fluconazole susceptibility is FLU1, identified in *S. cerevisiae*. However, no fluconazole-resistant clinical isolates have been found to overexpress this gene, suggesting it may not be clinically significant.

**Biofilm formation**

Biofilm formation is a critical mechanism that enhances *Candida*‘s resistance to azole antifungal drugs. *Candida* species have a remarkable ability to create biofilms, which are complex, multicellular communities encased in a protective extracellular matrix. Biofilm formation plays a significant role in the persistence of *Candida* infections and poses a substantial challenge in their treatment.

Within a biofilm, *Candida* cells are enmeshed in an extracellular matrix composed of polysaccharides, proteins, and other biomolecules. This matrix acts as a physical barrier, preventing the penetration of azole drugs into the deeper layers of the biofilm. As a result, the antifungal agents have limited access to the individual fungal cells, reducing their effectiveness. Even if some azole drugs manage to diffuse through the extracellular matrix, the dense structure of the biofilm hinders their penetration into the cells. The biofilm’s architecture limits drug diffusion, leading to lower intracellular concentrations of azoles within *Candida* cells, making it difficult for the drugs to reach their target sites effectively.

The biofilms utilize quorum sensing, a communication mechanism that allows cells to coordinate their behaviour based on cell density. Within biofilms, quorum sensing triggers changes in gene expression, leading to the activation of various stress responses and adaptive mechanisms. This allows *Candida* to adapt and develop greater resistance to azole drugs, further enhancing its survival within the biofilm.

**DISCUSSION**

The rise in azole resistance among *Candida* species has posed significant challenges in the effective management of *Candida* infections. This has prompted extensive research into understanding the complex mechanisms driving this resistance, involving intricate genetic, cellular, and biochemical processes. The resistance role of CDR, a member of the ABC family of drug transporters and MDR, which belongs to the MFS family, has been well documented by researchers. Mutations in these genes can potentially result in...
the development of pan resistant strains.\textsuperscript{69} The diverse genetic mechanisms driving azole resistance, primarily revolving around mutations in crucial genes like ERG11, ERG3, and ERG5. These mutations can lead to alterations in the structure and function of enzymes critical for ergosterol biosynthesis, consequently reducing the efficacy of azole drugs. Additionally, the altered drug transport mechanisms, particularly the overexpression of efflux pump genes like CDR1, CDR2, and MDR1, which actively expel azole drugs from the fungal cells, reducing their intracellular concentration and rendering the treatment less effective. There are various genes that belongs to the ABC and MFS transporter families in the different Candida species like \textit{C.tropicalis},\textsuperscript{69} \textit{C.krusei},\textsuperscript{70} \textit{C. glabrata},\textsuperscript{71,72,73,74} \textit{C.dubliniensis} \textsuperscript{75,76} and \textit{C.parapsilosis}.\textsuperscript{77} The comprehensive understanding of these intricate mechanisms not only underscores the challenges in treating azole-resistant Candida infections but also underscores the pressing need for the development of novel therapeutic approaches.

CONCLUSION

Significant advancements have been made in recent years regarding the control of azole antifungal resistance in \textit{Candida} through various mechanisms. Understanding the mutations that activate these factors and the mutations in the Erg11p gene, which is responsible for the azole target, may contribute to enhanced methods for predicting treatment failure with azoles in patients suffering from \textit{Candida} infections. Alterations in drug transport mechanisms, particularly the overexpression of MFS and ABC transporter genes, contribute to azole resistance in various \textit{Candida} species. Understanding these resistance mechanisms can aid in the development of strategies to combat azole-resistant fungal infections. The emergence of new technologies enabling comprehensive genome-wide analysis across various levels holds immense potential for gaining fresh insights into the regulation of antifungal resistance, and it promises to drive progress in this field in the years to come. To add on to this, understanding the mechanisms of action of biofilms and also the cellular processes that modulates resistance will also help to developing effective strategies to combat infections and antifungal resistance.

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Conflict of Interest

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Author contributions

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REFERENCES

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### Table 1: History of the most common triazole antifungal drugs

<table>
<thead>
<tr>
<th>Azole</th>
<th>Year</th>
<th>Major advantage</th>
<th>Major disadvantage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fluconazole</td>
<td>1990</td>
<td>Good oral bioavailability, easily tolerated by the body, effective in reaching the central nervous system, and demonstrates efficacy against yeast infections.</td>
<td>Shows restricted effectiveness against filamentous fungi, CYP450 interactions</td>
</tr>
<tr>
<td>Itraconazole</td>
<td>1992</td>
<td>Bioavailability variable, demonstrates efficacy against both filamentous and endemic fungi.</td>
<td>Bioavailability variable, CYP450 interactions</td>
</tr>
<tr>
<td>Voriconazole</td>
<td>2002</td>
<td>Strong efficacy against Candida, available in both injectable and oral forms.</td>
<td>Bioavailability variable</td>
</tr>
<tr>
<td>Posaconazole</td>
<td>2006</td>
<td>Active against zygomycetes</td>
<td>Only oral forms available, difficult to achieve therapeutic levels</td>
</tr>
<tr>
<td>Isuvaconazole</td>
<td>2015</td>
<td>Broad spectrum of activity, Good oral bioavailability, available in both injectable and oral forms.</td>
<td>Gastrointestinal disturbances, CYP3A4 interactions</td>
</tr>
</tbody>
</table>

### Table 2: ABC and MFS transporters in Candida species mediating azole resistance

<table>
<thead>
<tr>
<th>Candida species</th>
<th>ABC transporter genes</th>
<th>MFS transporter genes</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>C. albicans</td>
<td>CDR1, CDR2</td>
<td>MDR1</td>
<td>66, 67, 68</td>
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<tr>
<td>C. tropicalis</td>
<td>None</td>
<td>CtMDR1</td>
<td>69</td>
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<tr>
<td>C. krusei</td>
<td>CkABC2</td>
<td>None</td>
<td>70</td>
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<tr>
<td>C. glabrata</td>
<td>CgCDR1, PDH1, SNQ2</td>
<td>CgFLR1</td>
<td>71, 72, 73, 74</td>
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<tr>
<td>C. dubliniensis</td>
<td>CdMDR1</td>
<td>CdMDR1</td>
<td>75, 76</td>
</tr>
<tr>
<td>C. parapsilosis</td>
<td>CpCDR1</td>
<td>CpCDR1</td>
<td>77</td>
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