



Review Article

Calcium signaling pathway is involved in non-CYP51 azole resistance in *Aspergillus fumigatus*

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Abstract

The opportunistic fungal pathogen *Aspergillus fumigatus*, which is one of the primary airborne ascomycete pathogens and allergens worldwide, causes invasive fungal infections, which have high morbidity and mortality rates among immunosuppressed patients. The abuse of azole antifungals results in serious drug resistance in clinical therapy. Thus, a thorough understanding of the azole drug resistance mechanism and screening of antifungal agents with a novel mode of action and new drug targets are required to fight against drug resistance. Current studies suggest that there are three major azole resistance mechanisms in fungal pathogens, including changes of the drug target Cyp51, activation of drug efflux pumps and induction of cellular stress responses. Fungi must adapt to a variety of external environmental stressors to survive. These obstacles include stress to the plasma membrane after azole antifungal treatments, high temperature, pH variation, and oxidative stress. As a filamentous fungus, *A. fumigatus* has evolved numerous signal-transduction systems to sense and respond to azole stresses to survive and proliferate in harsh environmental conditions. Among these signal-transduction systems, the Ca^{2+} signaling pathway is one of the most important response systems, which has been verified to be involved in stress adaptation. In this review, we have summarized how the components of the calcium-signaling pathway and their interaction network are involved in azole stress response in *A. fumigatus*.

Key words: calcium signaling pathway, non-CYP51 azole resistance, *Aspergillus fumigatus*.

Introduction

In all eukaryotic cells, calcium is a second messenger that is involved in growth, development, secretion, transportation and stress response.¹ Under normal physiological conditions, the cytosolic free calcium ($[\text{Ca}^{2+}]_c$) concentration is strictly and precisely controlled by the complex of calcium signaling pathway that consists of various channels, transporters, pumps, sensors, and effectors located in the plasma membrane, intracellular storage organelles, and calcium buffering in the cytoplasm.^{2–4} In the unstimulated cell, cytosolic Ca^{2+} concentrations are maintained at a resting level between 50 and 200 nM, but their concentrations in the intracellular storage organelles are thousands of times higher.^{5,6} Upon stimulation, Ca^{2+} is rapidly released from

the intracellular Ca^{2+} pools. During this transient response, a variety of effectors are activated to adapt for environmental stimuli.^{2,7}

By mimicking a clinical, long-term azole exposure environment, stable itraconazole-resistant strains were successfully obtained from clinic-derived *Aspergillus fumigatus* isolates cultured on media containing high concentrations of itraconazole. Through a series of procedures, such as reverse genetics, site-directed mutations, agar plate susceptibility assay, broth microdilution for cross-resistance testing, and next-generation sequencing, new non-CYP51 mutations conferring azole resistance have been identified. Among these itraconazole-resistant isolates, hyperactivated cytoplasmic calcium responses were found

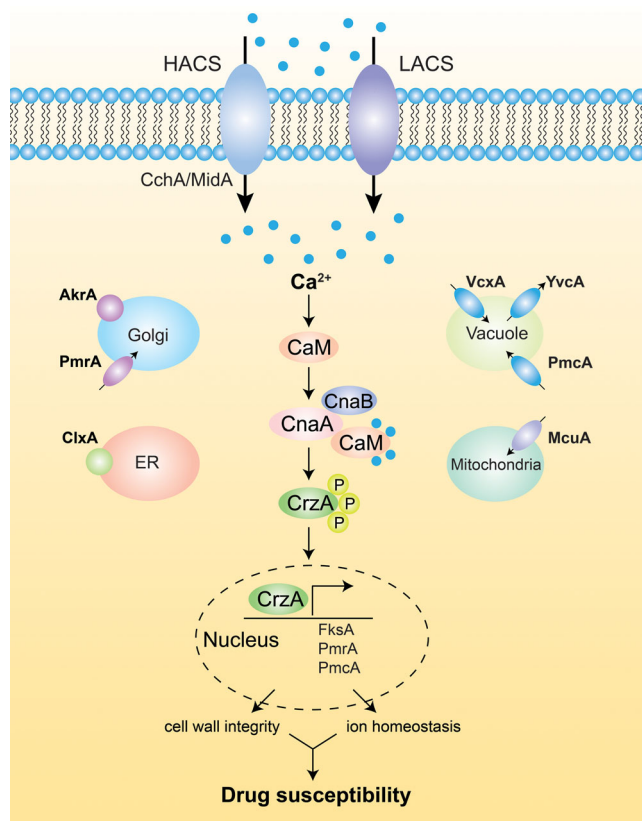


Figure 1. Schematic representation of calcium signaling pathway involved in azole stress. Calcium homeostasis is tightly regulated by various components in *A. fumigatus* including CchA/MidA on plasma membrane, PmrA in Golgi, ClxA in ER, McuA in mitochondria, VcxA, PmcA and YvcA in vacuole. Defects in calcium homeostasis lead to the retention of cytoplasmic calcium which enhances the calcineurin phosphatase activity by Ca^{2+} -calmodulin complex. The activated calcineurin complex dephosphorylates the calcineurin-dependent transcription factor, CrzA, and enables its translocation into the nucleus. CrzA activates the transcription of genes involved in the regulation of cell wall integrity and ion homeostasis and consequently affecting drug susceptibility. This Figure is reproduced in color in the online version of *Medical Mycology*.

to be contributed to azole-drug resistance.^{8,9} In this study, we summarize previously published papers and our findings, which indicate how the calcium-signaling pathway involves azole-drug resistance in fungal pathogens.

The complex of calcium signaling pathway is required for the cytosolic calcium homeostasis

Calcium uptake systems in the plasma membrane

In most fungal species, two different calcium uptake systems in the plasma membrane have been identified: the high-affinity calcium influx system (HACS) and the low-affinity calcium influx system (LACS) (Fig. 1). In the budding yeast *Saccharomyces cerevisiae*, the HACS consists of the ortholog of a α -subunit of the mammalian voltage-gated calcium channel Cch1 and a stretch-activated β -subunit calcium channel/regulatory protein Mid1. Both Cch1 and Mid1 proteins have been shown to be

necessary for Ca^{2+} influx, which acts as the major calcium entry route under low calcium availability conditions in *S. cerevisiae*.^{10,11} In comparison, when cells meet high environmental calcium availability, the LACS, which is primarily composed of mating factor-induced gene 1 (Fig. 1), will be activated to function as calcium channels in response to pheromone treatment.^{12,13} In the filamentous fungus *Aspergillus nidulans*, the calcium channel CchA and its regulatory subunit MidA form the HACS to facilitate calcium influx in low-calcium concentration environments. Therefore, the loss of *cchA* or *midA* results in decreased cytosolic Ca^{2+} , while the addition of extracellular Ca^{2+} can significantly rescue their growth defects, suggesting that the high-calcium environment could bypass the requirement of the HACS^{14,15}. Comparably, the opportunistic fungal pathogen *A. fumigatus* has a similar response to calcium environments but with a reduced calcium response compared to the model organism *A. nidulans*.¹⁶

Calcium release from intracellular calcium stores

In fungal cells, many lines of evidence indicate that the endoplasmic reticulum, Golgi apparatus, mitochondria, and vacuole function as intracellular calcium stores depending on the P-type Ca^{2+} transporting ATPases and calcium pumps located on these organelle membranes. The P-type Ca^{2+} transporting ATPases expend energy derived from ATP for transferring Ca^{2+} against the ion gradients. According to the tissue distributions and functions of pumps, nine different Ca^{2+} pumps in humans corresponding to at least 30 splice variants have been identified and classified into three groups: PMCA (plasma membrane Ca^{2+} -ATPase), SERCA (sacro/endoplasmic reticulum Ca^{2+} -ATPase), and SPCA (secretory pathway Ca^{2+} -ATPase).¹ In comparison, *S. cerevisiae* has human P-type Ca^{2+} transporting ATPases homolog Pmr1, which is a plasma membrane ATPase-related $\text{Ca}^{2+}/\text{Mn}^{2+}$ pump localized in the Golgi apparatus. The deletion of *S. cerevisiae pmr1* results in the retention of Ca^{2+} and Mn^{2+} in the cytoplasm, while deletion of *pmrA*, a yeast *pmr1* homolog in *A. nidulans*, leads to indistinctly decreased cytosolic calcium concentrations, suggesting that the function of Golgi-localized Pmr1/PmrA is not conserved across different species.^{17,18} A recent study reports that a lectin chaperone Ca^{2+} -binding protein-ClxA, a mammalian calnexin homolog, is involved in the fungal endoplasmic reticulum stress response by regulating Ca^{2+} homeostasis in *A. nidulans*. Inactivation of calnexin causes severe defects in hyphal growth and conidiation under the endoplasmic reticulum and high temperature stresses in *A. nidulans*, indicating that ClxA may mediate calcium release from internal calcium stores.¹⁹ Comparably, Ca^{2+} uptake into mitochondria plays a central role in cell physiology by stimulating ATP production, shaping cytosolic Ca^{2+} transients and regulating cell survival or death.¹ As intracellular calcium stores, mitochondria have been studied extensively in mammalian cells, while a recent study demonstrates

that the *A. fumigatus* genome encodes a putative mitochondrial homolog Ca^{2+} uniporter-McuA, which contributes to Ca^{2+} uptake into mitochondria and involves azole stress responses.^{20,21} In addition, the $\text{Na}^+/\text{Ca}^{2+}$ exchanger (mNCX) and the $\text{Ca}^{2+}/\text{H}^+$ exchanger (mHCX) primarily contribute to Ca^{2+} extrusion from mitochondria to cytosol.²² In addition, the vacuole, an important compartment in fungi, is crucial for adaptation to stress, endocytosis, autophagy, and pathogenesis and has also been recognized as the main organelle for storage and a Ca^{2+} sink that plays important roles for recycling intracellular calcium.^{2,23} For vacuolar storage of Ca^{2+} , two principal transporters have been identified: the lower-affinity vacuolar $\text{Ca}^{2+}/\text{H}^+$ exchanger-Vcx1 and the high-affinity Ca^{2+} -ATPase-Pmc1 in yeast *S. cerevisiae*.^{24,25} Deletion of *vcx1*, *pmc1*, or *vcx1/pmc1* in *S. cerevisiae* greatly affects the characteristics of Ca^{2+} responses. Ca^{2+} storage is greatly attenuated in the *vcx1* mutant during the transient elevation of cytosolic calcium (TECC) response.²⁶ In *S. cerevisiae*, Vcx1p is identified as a protein complex that is predominantly responsible for restoring cytosolic Ca^{2+} concentrations after brief challenge with high extracellular Ca^{2+} concentrations, while Pmc1p appears to be critical for long-term Ca^{2+} tolerance.²⁵ Although single deletion of *pmc1* shows no significant effect on Ca^{2+} storage during the TECC response, it greatly attenuates Ca^{2+} storage during the Ca^{2+} pulse.²⁶ In contrast, double knockout of *Cryptococcus neoformans* *pmc1* and *vcx1* genes impairs intracellular calcium transport, resulting in a significant increase in cytosolic calcium levels.^{27,28} However, Dinamarco et al. has proposed that *A. fumigatus* has three Pmc1 and four Vcx1 homologues, both Pmc1 homolog genes-*pmcA* and *pmcB* mutants in *A. fumigatus* show sensitivity to Ca^{2+} compared to the parental strain.²⁹ In addition to maintaining low cytosolic calcium, the vacuole also plays a vital role in releasing calcium into the cytoplasm in response to external stresses.³⁰ Yvc1p in *S. cerevisiae* and YvcA in *A. fumigatus*, which are homologs of mammalian transient receptor potential (TRP) proteins, have been identified as vacuole membrane-localized calcium channels contributing to cytoplasmic calcium fluctuation by releasing calcium from the vacuole into the cytoplasm. Although the expression of *yvc1* strongly depends on the cytosolic Ca^{2+} concentration in *S. cerevisiae*, the deletion of *yvc1* results in no significant alteration in cytosolic calcium under various stresses.^{26,31} Unlike *yvc1*, the *A. fumigatus* *yvcA* mutant shows a constant low level of intracellular calcium concentrations, suggesting that *yvcA* mediates cytoplasmic calcium homeostasis.¹⁶

Relationships between the functional calcium-signaling pathway and azole susceptibilities in fungi

Fungal pathogens have evolved elaborate mechanisms to sense varied environmental stresses, including drug stress, oxidative

stress, osmotic stress, and thermal stress.^{32–35} The calcium-based signaling system can alter the free Ca^{2+} concentration in the cytoplasm in response to environmental stimuli and physiological signals through regulating calcium channels or calcium transporters, Ca^{2+} -binding proteins, as well as other downstream proteins.³⁶ There are three different categories that we have summarized below, which show evidences for the functional calcium-signaling pathway involved in azole resistance.

Synergistic effect of inhibitors or blockers of calcium signaling pathway with antifungals

There are many reports documenting that non-antifungal compounds, such as amiodarone, cyclosporine (CsA), tacrolimus (FK506), rapamycin, the estrogen receptor antagonists-tamoxifen and toremifene, and certain calcium channel blockers exhibit antifungal activity alone or in combination with antifungal drugs through interference with the functions of these components.^{37–42} In both *S. cerevisiae* and *A. fumigatus*, azole efficacy is reduced by the addition of Ca^{2+} for fungal cells. Conversely, azole and polyene drug (amphotericin B) efficacy are enhanced by the addition of the Ca^{2+} chelator ethylene glycol tetraacetic acid (EGTA) and ethylene diamine tetra-acetic acid (EDTA).^{43–45} Moreover, the cytoplasmic Ca^{2+} level is able to be sharply increased by azole stimuli while the addition of EGTA results in remarkably decreased cytosolic Ca^{2+} concentrations in fungal cells, suggesting that the calcium influx response induced by azole is inhibited by EGTA.⁴⁴ In addition, the disruption of biofilms, which are associated with drug resistance by EDTA, has also been observed in *C. albicans*, indicating the importance of calcium homeostasis for fungal pathogens.⁴⁶ These data suggest that the altering extracellular Ca^{2+} concentrations are capable of influencing the antifungal activity of antifungal drugs.

CsA and FK506, inhibitors of the calcineurin activity via interaction with respective immunophilins, cyclophilin A, and FKBP12, have been shown to display a synergistic effect with fluconazole treatment against azole-resistant *C. albicans*.^{36,47} Moreover, inhibitors of calcineurin are also effective against *A. fumigatus* *in vitro* through a synergistic effect with echinocandin, caspofungin.^{48,49} In addition to those inhibitors of calcineurin, three structurally distinct inhibitors (fluphenazine, calmidazolium, and a W-7 analogue) of the Ca^{2+} -binding regulatory protein calmodulin are also capable of enhancing azole activity.⁴³ Verapamil, a phenylalkylamine class of calcium channels blockers that decreases Ca^{2+} concentrations, inhibits hyphal development and adhesion in *C. albicans*. Furthermore, verapamil and fluconazole have also been observed to exert combined effects, inhibiting *C. albicans* biofilm formation.⁵⁰ This evidence suggests that the inhibitors of the calcium-signaling pathway or calcium channel blockers have a synergistic effect along with azoles against azole-resistant isolates.

Loss of functional calcium signaling pathway components cause the alteration of azole susceptibility

In *Candida glabrata*, the loss of calcium channel *mid1* or *cch1* results in increased susceptibility to fluconazole compared to the wild type.⁵¹ The same phenomenon in *A. fumigatus* shows that compared to that of the parental wild type, the $\Delta cchA$ and $\Delta midA$ strains are more susceptible to various azoles, including voriconazole, ketoconazole and itraconazole due to decreased cytosolic Ca^{2+} concentrations.¹⁶ In addition, the blocking of the vacuole channel in *C. neoformans*, which transfers calcium from cytoplasm to vacuole, results in a continuous activated calcium signaling pathway due to cytosolic Ca^{2+} retention.²⁸ In contrast, in the *pmcA*, *pmcB* or *yvcA* mutants of *A. fumigatus*, there are no detectable changes of cytosolic Ca^{2+} concentration and susceptibility to antifungal agents, suggesting that redundant and complex calcium uptake channels or storages may exist in *A. fumigatus* for regulating Ca^{2+} homeostasis.²⁹ The retention of cytosolic Ca^{2+} could also be achieved in the *pmr1* mutant in *S. cerevisiae*, which causes continuous activation of the calcium-signaling pathway.⁵² Although there is no obvious alteration of cytosolic Ca^{2+} in the *A. fumigatus* *pmrA* mutation, the *mcuA* mutants display increasing in cytosolic Ca^{2+} , which cause azole resistance compared to the parental strain. Moreover, the addition of the Ca^{2+} chelator, EGTA, rescues the reduced azole susceptibility phenotype of the *mcuA* mutant.²⁰ These studies show that the alteration of cytosolic Ca^{2+} caused by the defects of calcium channels may affect the response to azole stress.

Defects of posttranslational modifications of main components in calcium signaling pathway result in increased azole susceptibilities

In fungi, upon stimulation by various external stresses, increased cytosolic Ca^{2+} first leads the Ca^{2+} sensor protein, calmodulin (CaM), to be activated through binding with calcium ions and subsequently activates the phosphatase calcineurin. In *S. cerevisiae*, as the central controller of the calcium signaling pathway, calcineurin dephosphorylates the transcriptional factor Crz1 (calcineurin-responsive zinc finger 1), leading to its nuclear translocation.⁵³ Crz1 lastly activates downstream genes, including the Ca^{2+} -ATPase genes *pmc1*, *pmr1*, *pmr2*, and the glucan synthase gene *fks2*, the feedback of which affects calcium homeostasis and cell wall integrity, by binding to calcineurin-dependent responsive elements (CDREs) within their promoter regions.⁵ It has been reported that the proper function and localization of Crz1 depends on dephosphorylation catalyzed by calcineurin, which serves as a phosphatase.^{54,55} In *S. cerevisiae*, the mutation of *cna1* or *crz1* enhances sensitivity to miconazole, itraconazole and terbinafine.⁴³ A similar phenomenon is observed in *A. fumigatus* *cnaA* or *crzA* mutants. In addition to azoles, CrzA also plays a role in mediating the paradoxical effects caused by caspofungin since the high concentration of

caspofungin elicits a spike in cytosolic Ca^{2+} and activates the dephosphorylated-form of CrzA, up-regulating the downstream targeted gene expression of chitin synthase to increase chitin synthesis.⁵⁶

In addition to the phosphorylation of the transcription factor CrzA, previous work showed that palmitoylation, which is a reversible lipid modification, plays a vital role in calcium regulation and drug susceptibility in *Aspergilli*. Palmitoyl transferase AkrA, a homolog in the filamentous fungus *A. nidulans*, is required for normal growth and sporulation under low extracellular calcium cultural conditions. In AkrA dysfunction mutants, there exists significantly decreased cytosolic free calcium response, induced by high extracellular calcium stress, tunicamycin, or the antifungal agent itraconazole compared to its reference strain.¹⁸ These data show that posttranslational modification may also be involved in cytosolic calcium homeostasis and crucial for regulating the cell response to antifungal drugs in *A. fumigatus*.

In summary, fungi must adapt to a variety of environmental stress obstacles to survive, while these obstacles include stresses upon antifungal treatments. *A. fumigatus*, a widespread saprotroph fungus in nature, has evolved numerous signal-transduction systems to sense and respond to azole stresses to survive and proliferate in harsh environmental conditions. Among these signal-transduction systems, the Ca^{2+} signaling pathway is one of most important response system, which has been determined to be involved in stress adaptations. Calcium homeostasis is usually dependent on the equilibrium between the uptake and release of calcium ions from the extracellular environment and intracellular storages. Therefore, the endoplasmic reticulum, Golgi apparatus, vacuole and mitochondria, which are major intracellular calcium storage compartments, play important roles in maintaining intracellular calcium equilibrium. The regulation of calcium homeostasis requires activation of the trimeric complex (CnaA-CnaB-CaM) to elicit downstream responses by regulating CrzA to modulate the gene expressions of the calcium channels. Current studies collectively demonstrate that the activation of the calcium signaling pathway induced by azole stress contributes to the survival of fungal cells during drug exposure. In contrast, the blocking of the calcium signaling pathway or the loss of the functional calcium signaling pathway components in *A. fumigatus* cause enhanced drug susceptibilities. Thus, effectively utilizing the inhibitors or blockers of the calcium signaling pathway can significantly enhance the pharmacology of limited antifungal agents, especially for non-CYP51 azole resistance isolates in clinical *A. fumigatus*.

Perspectives and clinical indications

The calcium-involved stress-response pathway might be used as new antifungal drug targets

Although calcineurin, a central component in the calcium signaling pathway, has been used as a drug target in the clinic,

currently available anti-calcineurin agents, such as FK506 and CsA, are not ideal for treating invasive fungal infections since these drugs have subsequent immunosuppressive side effects.⁵⁷ Butts et al. demonstrates that tamoxifen and toremifene exerts their anticryptococcal activity alone or in combination with fluconazole and amphotericin B by directly binding to the essential EF-hand protein calmodulin.³⁷ Moreover, the antifungal peptides PAF and AFP_{NN5353} exert their antifungal activity by influencing the cytosolic calcium concentrations.^{39,40} Therefore, these findings suggest that other components of calcium-involved stress-response pathway might be the promising targets for new antifungal drugs with low toxicity to the host.

The synergistic combinations of antifungals and calcium signaling inhibitors might be a compromising strategy

Invasive fungal infections caused by uncommon filamentous fungi, such as *Mucorales*, *Fusarium*, and *Scedosporium*, has increased in the last decades and generally difficult to treat due to primary resistance to many currently available antifungal drugs.^{58–60} There have been reports that synergistic effects of various antifungal combinations or antifungals and immunosuppressive agents (tacrolimus and sirolimus) have been noted *in vitro* against mucormycosis, and combinations of antifungals and antimicrobials or non-antifungal agents shows potential benefits in the treatment of fusariosis.^{61–64} As synergistic drug combination has been proved to be a valid therapeutic approach, antifungal drugs in combination with calcium signaling pathway inhibitors might be new way to fight against fungal drug resistance in prospectively clinical utilization.

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Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and the writing of the paper.

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