Echinocandin Resistance in *Aspergillus fumigatus* Has Broad Implications for Membrane Lipid Perturbations That Influence Drug-Target Interactions

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ABSTRACT: Echinocandin drugs target the fungal enzyme β-(1,3)-glucan synthase (GS), which is required for the synthesis of cell wall component β-(1,3)-D-glucan. They are first-line therapy for *Candida* infections but are increasingly used as second-line therapy for *Aspergillus* infections. Resistance to echinocandins has been mainly studied in *Candida* and occurs due to mutations in *FKS* genes encoding GS. In our recent report, we identified a novel mechanism of echinocandin resistance in *Aspergillus fumigatus*. We showed that caspofungin exposure modifies GS, rendering it insensitive to echinocandins. This mechanism of resistance involved alteration of the GS lipid microenvironment and was mediated via an off-target effect on mitochondria leading to increased reactive oxygen species (ROS). We hypothesized that caspofungin-induced ROS alters the lipid composition around GS, changing its conformation and making it insensitive to echinocandins. In this commentary, we review both *fks1*-dependent and *fks1*-independent mechanisms of echinocandin resistance in *A fumigatus*. We believe this new resistance mechanism is also conserved among *Candida* spp. with implications for drug tolerance and/or resistance. Furthermore, we propose that ROS acts as a signaling molecule regulating lipid biogenesis, which impacts the structure-function of membrane proteins with implications for other types of drug-target interactions.

KEYWORDS: echinocandin resistance, tolerance, lipid microenvironment

The mold pathogen *Aspergillus fumigatus* is a primary cause of invasive aspergillosis resulting in life-threatening infections among immunocompromised patients. Azole antifungal drugs, which target the ergosterol biosynthesis pathway, are first-line therapy for the treatment of *Aspergillus* infections and are fungicidal. In contrast, fungistatic echinocandins are second-line therapy for *Aspergillus* infections and are used in treating patients who are refractory to primary azole therapy. Currently, there are 3 Food and Drug Administration (FDA)-approved echinocandin drugs: caspofungin, micafungin, and anidulafungin. The rising global incidence of azole resistance in *A fumigatus* has prompted an expanded use of echinocandins as therapy to treat *Aspergillus* infections.[1] As drug resistance emerges, there is a need to better understand underlying mechanism(s).

## Known Mechanism of Clinical Echinocandin Resistance

Resistance to echinocandins is mostly observed in *Candida* spp., where it is known that mutations in hot-spot regions of *FKS* genes confer resistance.[2] These mutations cause amino acid substitutions in the target enzyme glucan synthase, which alter drug-binding affinity, leading to drug resistance and clinical failures. Mutations in *FKS* hot-spot regions confer cross-resistance to all echinocandin class drugs. Due to limited usage of echinocandins in treating *Aspergillus* infections, little is known about the mechanisms of echinocandin resistance in

A fumigatus. We recently reported the first clinical case of an echinocandin-resistant *A fumigatus* isolated from a patient with chronic pulmonary aspergillosis[3] and demonstrated that the isolate had a mutation in the conserved hot-spot region of the *fks1* gene, comparable to what is seen with *Candida* resistance.

A Novel Mechanism of Clinical Echinocandin Resistance

Among a pool of clinical isolates of *A fumigatus* isolated from patients with chronic pulmonary aspergillosis who failed echinocandin therapy, there were many isolates that did not contain mutations in the *fks1* gene, suggesting the presence of a novel resistance mechanism. We recently described this novel resistance mechanism[4] and believe that it could become more common as echinocandin therapy for aspergillosis increases. In our report, we detected a modified form of glucan synthase, which was formed in cells during exposure to caspofungin and also showed a dose-dependent insensitivity to echinocandins in vitro. Importantly, caspofungin exposure during growth induced an altered lipid composition in the plasma membrane resulting in a change in the microenvironment of glucan synthase that rendered it drug insensitive. Finally, we identified an off-target effect of caspofungin in cells involving mitochondrial reactive oxygen species (ROS) production, which we proposed acts as a cellular signal for altered lipid biosynthesis. Collectively, these results have led us to propose a model
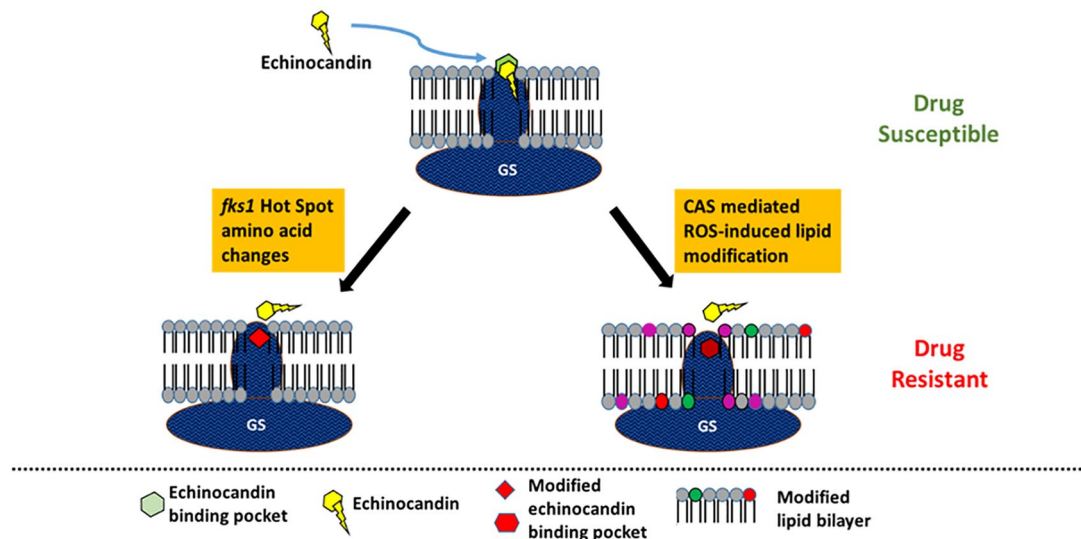
Figure 1. Diagrammatic representation of fks mutant-dependent and mutant-independent mechanisms of echinocandin resistance in *A fumigatus*. Source: Adapted from Satish et al.[4]

whereby caspofungin induces increases in mitochondrial ROS leading to changes in lipid composition in the membrane microenvironment of glucan synthase. The new lipid milieu alters the enzyme drug-binding affinity, either directly or indirectly, which ultimately leads to drug resistance.

In summary, both *fks1* mutation-dependent and *fks1* mutation-independent mechanisms of echinocandin resistance can lead to clinical failure of *Aspergillus* infections. The 2 kinds of clinical resistance to echinocandins are summarized in Figure 1.

Off-Target Effect of Caspofungin-Mitochondrial ROS Production

Although echinocandins are suggested to act from the outside of the cell, one study in *Candida albicans* showed that caspofungin is taken up by cells through a high-affinity facilitated-diffusion transporter.[5] Since caspofungin enters the cytoplasm, it allows for possible off-target interactions of the drug within the cell. A study to understand physiological mechanisms of caspofungin-induced cell death in *C albicans* showed that the drug also kills cells by causing both cellular apoptosis and necrosis.[6] Furthermore, the cells showed ROS production on exposure to caspofungin. Another study in *C albicans* showed that exposure to caspofungin triggers activation of glutathione reductase (GLR1), superoxide dismutase (SOD2), and mitochondrial processing protease (MAS1) genes, all of which are known to be associated with detoxification of cells during periods of oxidative stress.[7] Furthermore, in vitro studies have shown that addition of ROS scavengers, such as *N*-acetyl-ʟ-cysteine and Vitamin C, to *C albicans* cells treated with echinocandins made the cells less susceptible to caspofungin.[8]

In our study, we measured ROS production in *A fumigatus* strains using a fluorescent dye-based assay and observed that

only caspofungin, but not other echinocandins, induced prominent ROS production in cells.[4] Furthermore, addition of mitochondrial inhibitor antimycin A significantly reduced ROS levels, indicating that caspofungin-induced ROS is mitochondrial in origin. Importantly, neutralizing ROS with scavengers blocked induction of downstream drug resistance. Most recently, preliminary data obtained in *C albicans, Candida glabrata*, and *Candida auris* also showed similar caspofungin-specific mitochondrial induction of ROS effects with lipid alterations that alter target properties (data not shown). The implications of this work, which are ongoing, are significant for potential cell tolerance and drug resistance in *Candida*.[9]

ROS as a Signaling Molecule Altering Lipid Composition in Fungal Plasma Membrane

ROS, a group of chemical species comprising free radicals such as superoxide, hydroxyl radical, and singlet oxygen, and non-radical species such as hydrogen peroxide, show chemical reactivity that is not observed with molecular oxygen. Historically, they are known to be detrimental for cell survival, as they have the capacity to damage cellular components such as nucleic acids, proteins, and lipids. Classically, ROS are viewed as molecules employed by host immune cells for fighting microbial pathogens. However, more recent studies indicate that ROS can also be beneficial and are involved in growth and differentiation of cells.[10] In addition, they also act as secondary messengers and modify various signaling molecules that could help maintain normal physiologic functions and even promote cell survival.[11-14] ROS can react with cysteine residues in proteins and oxidize them, causing structural changes within the proteins and altering their functions. This in turn can affect downstream transcription, phosphorylation, and other important signaling events.[15-17] Cancer cells maintain a high intracellular level of ROS, which favors cell proliferation via several

pathways including mitogen-activated protein kinases (MAPKs).[16,18] In our study, we report a similar beneficial role of ROS in *A fumigatus* in the presence of drug. Our model suggests that enhanced ROS production induces a modified lipid composition with increased abundance of specific lipids (dihydrosphingosine and phytosphingosine) around glucan synthase, altering enzyme conformation and leading to echinocandin resistance.[4] How ROS brings about increased abundance of these lipids is yet to be understood. We hypothesize that ROS acts as a signaling molecule enhancing transcription of certain lipid biogenesis genes. We also speculate that clinically resistant isolates of *A fumigatus*[4] are genetically more sensitive to changes in ROS levels, perhaps due to mutation(s) in lipid biogenesis genes or ROS-sensitive transcription factors. This hypothesis is being investigated in greater detail because of its clinical significance.

Role of Plasma Membrane Lipids in Drug Resistance

Over the last several years, the role of lipids in development of microbial drug resistance has gained considerable attention. Numerous studies have reported that changes in plasma membrane lipid composition can affect the localization and functioning of key membrane proteins, including those involved in drug binding and trafficking.[19] The concept of modulating lipid composition to alter protein function has gained traction and is referred to as membrane lipid therapy.[19] Sphingolipids form an important component of fungal cell membrane, and in the past decade, several studies have focused on specific interactions between sphingolipids and membrane proteins. The 3´-hydroxyl group and the amide nitrogen in sphingolipids represent hydrogen-bond donors and acceptors, allowing these sphingolipids to form a network with other sphingolipids.[20] This constrained intramolecular hydrogen-bonded network leads to a reduction in the molecular area per sphingolipid, making them rigid and altering the membrane fluidity. In addition, sphingolipid-based regions have increased membrane thickness.[21] The abundance of polar sphingolipid head groups in these regions allows for changes in the interaction between transmembrane domains of membrane proteins and sphingolipids.

The importance of sphingolipids in normal cell function has implications for cancer therapy, drug resistance, and immune presentation. About 2 decades ago, researchers first showed that standard of care treatments, such as chemotherapeutics and radiation, modulate sphingolipid metabolism to increase endogenous ceramides, which kill cancer cells.[22] Strikingly, resistance to these treatments has also been linked to altered sphingolipid metabolism, favoring lipid species that ultimately lead to cell survival. As cancer cells routinely display increased growth properties and escape from cell death, it has been suggested that enzymes involved in sphingolipid synthesis or catabolism may be altered in cancer cells.[23] Sphingolipids also have implications in development of drug resistance in

fungal species. In one study, laboratory and clinical strains of *C glabrata* showed an interesting phenotype of resistance to caspofungin but sensitivity to micafungin.[24,25] These "CRS-MIS" strains showed elevated dihydrosphingosine and phytosphingosine levels, and the study hypothesized that these changes in sphingolipids differentially modulated the binding affinities of caspofungin and micafungin to their target. Our study also found increased sphingolipids in the microenvironment of glucan synthase enzyme, altering its conformation and binding affinity to echinocandins, eventually leading to resistance.[4] A recent study in *C glabrata* emphasized the importance of phosphatidylinositol 3,5-bisphosphate, a low abundance lipid molecule, in the maintenance of cell wall chitin, caspofungin tolerance, survival within host cells, and virulence.[26] Another study in *C auris*, an emerging pathogenic fungus, showed higher abundance of sphingoid bases in its plasma membrane, including higher levels of phytosphingosine compared to *C albicans* strains.[27] This study also suggests that increased sphingolipid content may be related to increased resistance to antifungals typical of *C auris*. The authors mention that these lipids are important for the assembly of membrane platforms containing drug efflux pumps. Collectively, there is an increasing recognition for the important role of lipid composition in microbial resistance. Nevertheless, the role of altered lipid composition is clearly complex and requires further investigation.

In conclusion, we have identified a novel mechanism of echinocandin resistance in *A fumigatus* and have proposed that it is relevant to other fungal species and may be conserved at a higher level. The clinical impact of this mechanism is likely to become more significant, as the number of patients with *Aspergillus* infections being treated with echinocandins expands. For *Candida* spp. where echinocandins are already used as first-line therapy, the role of this mechanism in tolerance and potential drug escape is just emerging.

Author Contributions
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