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Change in Expression of Genes Involved in the G-Protein Signaling Pathway (GP-SP) is Associated with Voriconazole-Resistance (VCZ-R) in *Aspergillus* Species

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Authors' contributions

This work was carried out in collaboration between all authors. Author SKN designed the study, did the literature search, performed the statistical analysis, wrote the protocol, and the manuscript. Authors LF and WW performed majority of the experiments, did a literature search and contributed to the final analyses of the study. All authors read and approved the final manuscript.

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ABSTRACT

Background: Invasive infections due to *Aspergillus* species continue to be associated with a significant morbidity in immuno-compromised patients. Despite the availability of several azoles [isavuconazole (ISZ), posaconazole (POS), voriconazole (VCZ) and itraconazole (ITZ)], mortality remains high. Studies from various cancer and transplant centers around the world have reported the emergence of azole-resistance in clinical and environmental isolates of *Aspergillus fumigatus* and *Aspergillus flavus*. The major mechanism of high-level azole- resistance in *Aspergillus* species reported so far is mutation and/or overexpression of target site namely cyp51A, that encodes lanosterol demethylase of the fungal cell wall. However, some azole-resistant isolates have not exhibited either of these mechanisms, suggesting other novel non-cyp51 related mechanisms of triazole- resistance.

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Aim: To evaluate the possible role of G-protein signaling pathway genes in VCZ-R in Aspergillus species.
Materials and Methods: CLSI based susceptibility, and cyp51 gene-specific PCR experiments were performed on wild type and specific mutant strains of *Aspergillus* species to analyse the phenotypic alterations and changes in triazole-susceptibility pattern in *Aspergillus* species.
Results: Voriconazole inhibits conidiation in A. flavus, possibly through its effect on several genes involved in the GP-SP. Mutations or changes in expression of these genes contribute to VCZ-R in A. flavus. Loss of conidiation and pigmentation with switch to pure vegetative growth, exclusively by hyphal elongation, is associated with VCZ-R in A. flavus.
Conclusion: Our results suggest that VCZ inhibits conidiation by targeting one of the critical genes in the G-protein pathway and specific alterations in these genes likely lead to loss of conidiation and VCZ-R in *A. flavus*. Cross resistance to other triazoles including POS and ISZ need to be tested as well. Based on our data we propose to continue our studies on G-protein pathway genes involved in antifungal drug resistance in Aspergillus species. This pathway needs to be further explored not only for its possible contribution to VCZ-R but also to delineate its role in pathogenesis and also as a potential antifungal drug target.

Keywords: Aspergillus; antifungal drug resistance; voriconazole; posaconazole; G- protein signaling pathway; brlA; fluG; pkaC; flbA; fadA; mutation; overexpression; VCZ-resistance; triazole resistance.

1. INTRODUCTION AND RATIONALE

Invasive infections due to *Aspergillus species* continue to be associated with a significant morbidity in immune-compromised patients. Despite the availability of several azoles [isavuconazole (ISZ), posaconazole (POS), voriconazole (VCZ) and itraconazole (ITZ)], mortality remains high. Studies from various cancer and transplant centers around the world have reported the emergence of azole-resistance in clinical and environmental isolates of *Aspergillus fumigatus* and *Aspergillus flavus* [1,2]. The major mechanism of high-level azole-resistance in *Aspergillus species* reported so far is mutation and/or overexpression of target site namely *cyp*51A, that encodes lanosterol demethylase of the fungal cell wall [3,4]. However, some azole-resistant isolates have not exhibited either of these mechanisms, suggesting other novel non-*cyp*51 related mechanisms of triazole- resistance.

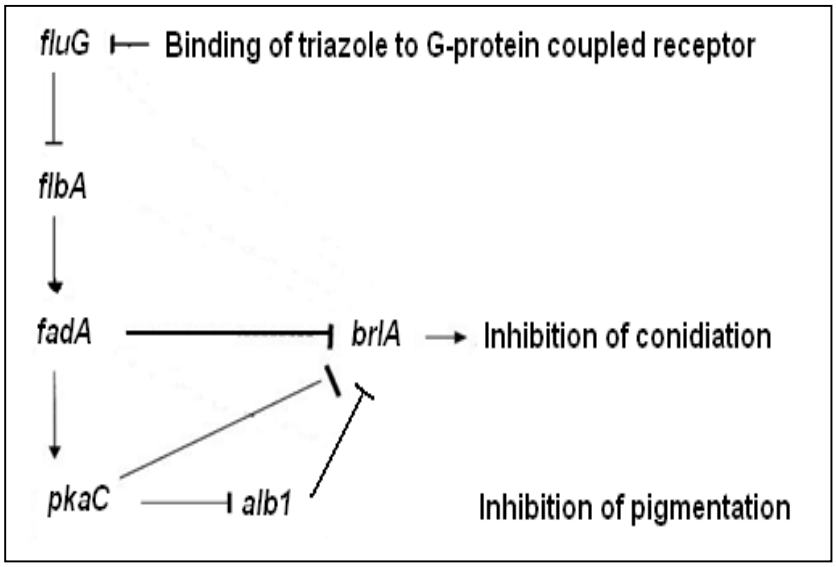

Fig. 1. Proposed model of interference of conidiation & pigmentation by triazoles in *A. flavus* (adapted from Keller and Calvo et al. Ref: [12,13])

(+) positive regulation; (-) negative regulation; fluG, early developmental regulator; flbA, regulator of G-protein signaling; fadA, α subunit of G-protein; pkaC, catalytic subunit of PKA; brlA conidiation -specific transcription factor; alb1, pigmentation gene

The recent description of a G-protein signaling pathway has for the first time established a direct linkage between growth, virulence, melanin pigmentation and conidiation in *Aspergillus spp*. A critical advance in this field was the recognition of the presence of a G-protein signaling pathway in *A. nidulans*. The present consensus is that this pathway mediates conidiation and is affected by secondary metabolites in the environment [5-8]. Although it is plausible that several different signaling pathways function in *Aspergillus spp*. the G-protein signaling pathway has been shown to be a dominant regulator of conidiation in several closely related *Aspergillus* spp.

As studies have shown a link between G-protein signaling pathway, conidiation, hyphal growth and virulence, we studied several GP-SP mutants to evaluate their role in phenotypic trait modifications and their contribution to VCZ-R in *Aspergillus species.*

2. MATERIALS AND METHODS

Several G-protein signaling pathway mutant strains were obtained from Fungal Genetics Stock Center, [*A. flavus* (Δ*flu*G and *fad*^{G42R}) and the nonpathogenic model fungus *A. nidulans* (Δ*flu*G, Δ*flb*A and Δ*brl*A)], were plated on Czapek Dox agar for 72h at 37ºC and observed for growth rate, pigmentation and conidiation followed by antifungal susceptibility testing. Fresh cultures were regrown, conidial suspensions were prepared and antifungal susceptibility testing was performed per CLSI M38-A2 guidelines [9]. The antifungal compound used in the study, namely, VCZ, was obtained as pure powders from Pfizer Pharmaceuticals (New York, NY, USA). and was stored at room temperature. The antifungal powder was dissolved in sterile water, divided into aliquots of 200 µL each, and was stored at − 20 °C until further use. The stock was then used to prepare the required concentrations of drug solutions for MIC testing (according to CLSI M38 A2 methodology). MIC was defined as the lowest concentration of the antifungal drug that resulted in 100% visual inhibition of growth after 48 h of incubation at 35°C. The minimum inhibitory concentrations of the various mutant strains were compared to that of the parent strains. Polymerase chain reaction, using gene specific primers, was performed to amplify *cyp*51 A and B genes to detect target site (gene encoding lanosterol demethylase, the target enzyme) in all mutant strains. Fresh cultures were inoculated into PYG broth for 48 h, hyphal mats extracted, and were subjected to DNA extraction using the Qiagen kit (Valencia, CA, USA) essentially following manufacturer-suggested protocols. The complete transcriptional unit (including the promoter sequence) of *cyp* 51A gene was amplified using gene-specific primers (Forward primer: 5′-ctcatcaactcttcatcactgcaactctaatcctc-3′; Reverse primer: 5′ gcactagttacacctatacggatcacacc-3′). Negative controls with no DNA were included with each amplification reaction. The polymerase chain reaction (PCR) products were then purified and sequenced at the Applied Genomic Technology Center (AGTC), Wayne State University, for identification and validation of mutations in the DNA of the *cyp* 51gene.

3. RESULTS AND DISCUSSION

On phenotypic examination, all mutant strains demonstrated loss of conidiation and pigmentation except for Δ*pkaC* that showed hyperpigmentation. Susceptibility testing with VCZ showed an increase in MIC by 2 to 128-fold in all mutant strains tested, as compared to wild type *A. flavus*, suggesting a likely role of this pathway in VCZ-R (Table 1). Importantly, none of the mutant strains tested demonstrated any mutations or overexpression of target site, *cyp*51A/B. Our observations indicate that mutations, overexpression, and/or deletion of critical components (regulatory proteins vis *pka*C or other critical genes responsible for conidiation and hyphal growth) of the GP-SP were associated with VCZ-R in *Aspergillus species*.

A cyclic AMP-dependent protein kinase (*pka*C) has been implicated in the regulation of both the pigmentation (*alb*1) and the conidiation pathways (*brl*A) in *A. nidulans, A. fumigatus* and *A. flavus*. The strongest evidence to support a genetic connection between *pka*C and other members of the G-protein signaling cascade in fungi has been through mutagenesis, cAMP /PKA activity studies and pathway studies detailed in *S. cerevisiae* and *A. nidulans* (Fig. 1) Inactivation of adenylyl cyclase decreases intracellular concentrations of cAMP and PkaC. Inhibition or deletion of *pka*C (a catalytic subunit of protein kinase) leads to overexpression of *flb*A (*a* regulator of G-protein signaling), loss of function of *fad*A (encodes an α-subunit of heterotrimeric G-protein) and overexpression of *brl*A (final conidiation-specific transcription factor), resulting in hyper conidiation. On the other hand, *pka*C overexpression mutants demonstrate *brl*A

Table 1. Susceptibility profile of various G-protein pathway mutant strains

G-protein pathway mutant strains	MIC of VCZ (mcg/ml)*	VCZ-susceptibility
A. flavus parent	0.125	Susceptible
*A. flavus Δflu*G	1	Resistant
A. flavus fad ^{G42R}	4	Resistant
A. flavus ΔbrlA	4	Resistant
A. nidulans parent	0.125	Susceptible
*A. nidulans Δflu*G	1	Resistant
*A. nidulans Δflb*A	8	Resistant
*A. nidulans Δfad*A	32	Resistant

**MIC interpretation of susceptibility (reference: [9])*

inhibition and complete loss of conidiation with a switch to vegetative reproduction by hyphal extension. As described above, changes in cAMP and PKA levels affect melanin synthesis as well. Interestingly, *pka*C deletion mutants (Δ*pka*C) overexpressed both *alb*1 and *brl*A resulting in rapid growth, hyper conidiation and increased pigmentation [9-16].

G-protein signaling pathway is a likely target for VCZ. Voriconazole inhibits conidiation in *A. flavus*, possibly through its effect on several genes involved in the GP-SP. Mutations or change in expression of these genes contributes to VCZ-R in *A. flavus*. Loss of conidiation and pigmentation with switch to pure vegetative growth, exclusively by hyphal elongation, is associated with VCZ-R in *A. flavus*. Our results suggest that VCZ inhibits conidiation by targeting one of the critical genes in the G-protein pathway and specific alterations in these genes likely lead to loss of conidiation and VCZ-R in *A. flavus*. Cross resistance to other triazoles including POS and ISZ need to be tested as well.

4. CONCLUSION

It is plausible that several different signaling pathways function in *Aspergillus spp*. the G-protein signaling pathway has been shown to be a dominant regulator of conidiation in several closely related *Aspergillus spp.*

Although more than one signaling pathway might be involved in conidiation, published literature has clearly established the GP-cAMP/PKA-SP as the dominant pathway that regulates conidiation in *A. nidulans*, *A. fumigatus* and *A. flavus*. The final step in the pathway involves activation of a conidiation transcription factor (*brl*A). Mutations or deletions involving critical genes upstream in the pathway affect *brl*A and interfere with conidiation.

Inhibition of the conidial transcription factor *brl*A resulted in loss of conidiation and switch to vegetative growth by hyphal elongation. It has been shown that deletion of *flu*G, an early acting developmental regulator in *A. flavus,* is associated with loss of conidiation and change in growth pattern in *A. flavus*, confirming the role of this pathway in conidiation. Also, deletion of positive regulators of *brl*A (Δ*flu*G, Δ*flb*A), overexpression of a common regulator *pka*C or dominant activating mutations in *fad*A (repressor of *brl*A), namely *fadA*G42R resulted in complete loss of conidiation in *A. nidulans*. Conidiation was restored by forced *brl*A overexpression in these mutants. Experiments have confirmed that *brl*A is directly involved in conidiation, is positively regulated by *flu*G and *flb*A and is negatively regulated by *fad*A and *pka*C.

Interestingly, during our experiments we observed that exposure of *A. flavus* to VCZ at ≥ 2 mcg/ml resulted in loss of both conidiation and pigmentation, a switch to vegetative growth by hyphal elongation and decreased susceptibility to VCZ. Our in *vitro* studies comparing the efficacy of azoles using conidia or hyphal masses as inoculums show that hyphae are more resistant to antifungal killing compared to conidia. These observations suggest a link between conidiation, pigmentation and VCZ-R and suggests that VCZ targets the conidiation and/or the pigmentation pathway in addition to its known inhibition of CYP51A.

Based on our data and data from Calvo et al., we propose to continue our studies on G-protein pathway genes involved in antifungal drug resistance in *Aspergillus species.* This pathway needs to be further explored not only for its possible contribution to VCZ-R but also as a potential antifungal drug target.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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