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Research Article

In vitro Melanin Inhibition and/or *alb*1 Mutations Modify Azole Susceptibility in *Aspergillus flavus*

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Abstract

Background: Melanin pigmentation is known to contribute to pathogenicity in *Aspergillus* species. However its role, if any in triazole susceptibility is unknown.

Aim: To investigate the *in vitro* fungicidal activity of the combination of melanin inhibitors (MIs) and voriconazole (VCZ) against voriconazole susceptible (VCZ-S) and resistant (VCZ- R) *A. flavus* and (ii) to investigate the association of *alb*1 gene alterations and/ or conidiation and triazole susceptibility in *A. flavus*

Methods: Fresh conidial suspensions were prepared from *A. flavus* cultures grown on SD agar and exposed to melanin inhibitors (MIs) namely tricyclazole (TCZ) and tropolone (TRO). MIC, FICI (fractional inhibitory concentration index), viability and time kill assays were performed using MIC and sub-MIC of TCZ, TRO and VCZ in RPMI using CLSI M-38A2 protocol. The fungicidal activities of MIs alone and in combination with VCZ were determined followed by PCR-based amplification of *alb*1 (gene encoding melanin) and gene transformation/knock-out experiments.

Results: Exposure to TCZ or TRO resulted in loss of pigmentation; TCZ (256 mcg/ml) and TRO (8 mcg/ml) demonstrated intrinsic antifungal activity against both wild-type (WT) and VCZ-R *A. flavus*. Susceptibility testing, viability and time kill assays using sub-MICs of MIs plus VCZ demonstrated synergism against WT and VCZ-R *A. flavus* at 24h and 48h with FICI ≤ 0.5. Alterations in *alb*1 of *A. flavus*, resulted in loss of pigmentation, normal conidiation and hyper susceptibility to VCZ.

Conclusions: Both TCZ and TRO demonstrate synergistic activity with VCZ against VCZ-R *A. flavus*; melanin inhibition, alterations in *alb*1 and conidiation modify triazole susceptibility in *A. flavus*.

INTRODUCTION

Invasive aspergillosis (IA) is a life threatening infection in immune compromised patients, especially following hematopoietic stem cell transplantation and graft versus host disease. Despite the availability of various antifungal agents, the mortality rate approaches 80 % and clearly new therapeutic approaches are needed [1]. Voriconazole (VCZ) is currently the drug of choice for management of IA. It is also being widely used as both empiric and prophylactic therapy around the world. Such widespread use of VCZ has led to the emergence of non-fumigatus *Aspergillus* that are intrinsically resistant to triazoles.

It has also resulted in the emergence of voriconazole resistance (VCZ-R) that could devitalize this agent and other triazoles (cross resistance) against IA. In fact triazole reports of TR in *Aspergillus* spp. continue to emerge in cancer and transplant centers around the world. It may evolve during triazole therapy in clinical strains or *de novo* from environmental isolates [2]. Several refractory clinical syndromes such as chronic granulomatous sinusitis, keratitis, myositis, endocarditis, spondylodiscitis, pneumonia, cholangitis, cutaneous aspergillosis, wound infections, osteomyelitis and disseminated infections caused by *A. flavus* have been reported in recent years with poor outcome, attributed to undiagnosed triazole-resistant IA [3].

Combination therapy is one approach that can be used

to improve the efficacy of antimicrobial therapy [4]. Most combination studies focus on currently available antifungal agents that have complementary targets within the fungal cell (polyenes plus triazoles or echinocandins) [4-6], whereas others have evaluated the use of non-antifungals such as immune factors, calcineurin inhibitors, statins, neutrophils etc. in combination with available antifungal agents [8-13]. The potential advantages of using combination therapy include broad spectrum of activity, greater potency, reduced toxicity and better efficacy.

Aspergillus flavus conidia are the first and foremost structures encountered by the human host, are 3-5 microns in diameter and are hydrophobic with yellowish green pigmentation. At the initial phase of fungal-host interaction, the conidial surface components, including melanin, play an integral role in evasion of the innate and acquired immune defense mechanisms [14]. Most importantly, melanin has been shown to contribute to fungal invasion, evasion and virulence in several pathogenic fungi including *Aspergillus species*, *Cryptococcus* and *Exophiala dermatitidis* [15-17]. Studies in *Aspergillus fumigatus* have demonstrated the presence of both di-hydroxyphenylalanine (DOPA) and di-hydroxynaphthalene melanin (DHN-M) pathways [18,19]. Chang et al., have performed knock-out studies of genes involved in the DHN-melanin pathway in *A. flavus*. Their studies revealed that although 6 genes were involved in the DHN-melanin synthesis pathway, only one gene which encodes a polyketide synthase, *alb*1, was confirmed to be responsible for melanin pigmentation in *A. flavus* [20]. Earlier *in vivo* studies have suggested the possible role of melanin in virulence and pathogenesis of *A. fumigatus* [21,22]. However none of the studies have evaluated the *in vitro* efficacy of melanin inhibitors (MIs) against *A. flavus* or investigated the role of *alb*1 gene alterations in VCZ-R in *A. flavus*. In this study we evaluated [1] the *in vitro* efficacy of MIs alone and in combination with VCZ against VCZ-susceptible and VCZ-R isolate of *A. flavus* and [2] studied the contribution of melanin pigmentation pathway to VCZ-R in *A. flavus*.

MATERIALS AND METHODS

Antifungal drugs, melanin inhibitors and fungal strains

Voriconazole was obtained as a pure powder from Pfizer Pharmaceuticals (New York, NY, USA), dissolved in dimethyl sulphoxide (DMSO) to make a stock solution of 1g/L and stored as 0.25 ml aliquots at –20ºC. Frozen stocks of the antifungal agents were thawed at room temperature and used within 24h. Voriconazole-R isolates of *A. flavus* used in this study (n=5) were selected, from a collection (n=120) of clinical specimens cultured from patients admitted to the Detroit Medical Center over a 5-10 year period. These isolates were sub-cultured in SD agar through 3-4 generations in the absence of VCZ, to check for persistence of VCZ resistance. Five of these isolates were then tested for *cyp*51A mutations and were found to have K197N, Y132N, T469S, D282E, M288L mutations (R100, R101, R102, R103, R104) The original cultures were again sub-cultured in Sabouraud dextrose agar (SDA) to check for stability, purity and viability. Working cultures were maintained on SDA slants at 4ºC. For long term preservation of the cultures, conidial suspensions were prepared in glycerol 25% v/v and stored at –80ºC. Melanin inhibitors namely Tropolone (TRO) and Tricyclazole (TCZ) were purchased from Sigma-Aldrich, stored at 4°C and fresh working suspensions were prepared in PYG medium (peptone, yeast and glucose) when needed. The final PYG based conidial and drug suspensions had negligible amounts of DMSO that ensured that DMSO based sporulation/melanin inhibition did not interfere with our test results. The effects of DMSO on sporulation and pigmentation were seen only at levels >10,000 ppm or 5-10%, when it was considered as an anti-aflatoxin agent in agriculture [23,24]. Our final working solution with PYG liquid medium had DMSO concentrations that were negligible (<0.001%).

MIC determination

Conidial suspensions from 6 day old *A. flavus* cultures were prepared, standardized by hemocytometry and used as inocula (2 X 10^4 /ml) for susceptibility testing. MICs of VCZ, TRO and TCZ for the 5 isolates of *A. flavus* were determined by the CLSI M38-A2 methodology using liquid PYG medium. MIC was defined as the lowest concentration of drug that resulted in 100% visual inhibition of growth. Drug concentrations ranging from 0.125 to16 mcg/ml (VCZ) or 1 mcg/ml to 512 mcg/ml (TCZ and TRO) were used for MIC determinations. Each MIC determination was performed in triplicate and repeated.

Fractional inhibitory concentration index determination (FICI)

The *in vitro* susceptibility of *A.flavus* to two-drug combinations of either TRO or TCZ with voriconazole was evaluated using the Fractional inhibitory concentration index (FICI) method. The FICI was determined with a checker board method in a microtiter plate using the M38-A technique. Pairwise combinations of the required concentrations of antifungal drugs A and B were prepared in 2-fold increments in RPMI-1640 medium. Appropriate drug-free controls were included. To each well 100 microliters of the conidial suspension (2X 10^4 conidia /ml) was added. The plate was incubated at 35ºC for 48h and the MIC was determined. Results from FICI determination, were confirmed using the previously described 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT)-based spectrophotometric assay (results not included). The FICI was then calculated using the following formula: FICI= (Ac/Aa) + (Bc/Ba) where Ac and Bc are the MICs of drugs A and B in combination whereas Aa and Ba are the MICs of drugs A and B respectively. Drug interaction was defined as synergistic (FICI < 0.5), no interaction (FICI >1 but <4) or antagonistic (FICI >4), based on results.

Kill-curve studies to evaluate the fungicidal activity of MIs

The fungicidal activity of either TCZ or TRO alone and in combination with VCZ against VCZ-R *A. flavus* isolates was determined by kill-curve experiments. Conidial suspensions (1X 10^6 conidia/ml) were incubated in the presence of sub-MIC (Minimum Inhibitory Concentration) of either TCZ (32 mcg/ml), TRO (2 mcg/ml) or VCZ (2 mcg/ml) alone or in combination. At 0, 6, 18, 24 and 48 hours, 0.1ml aliquots of the suspension were removed, diluted to obtain 10-10^3 dilutions, 0.1ml aliquots were spread on SDA plates and incubated at 35ºC for 24 h and the

number of CFU/ml was determined. Kill-curves were constructed by plotting mean $\log_{10}$CFU/ml against the time of exposure of conidia to various concentrations of VCZ, TRO and TCZ.

Melanin inhibition

Fresh conidial suspensions of wild type *A. flavus* and the VCZ-R strains were inoculated in SDA plates containing either TRO (4 mcg/ml) or TCZ (64 mcg/ml) and incubated at 35ºC. At 72 hours both macroscopic and microscopic examination of the colonies was performed.

Melanin inhibition by interference with *alb*1 gene

As a first step in exploring the pigmentation pathway in wild type *A. flavus*, we characterized the nucleotide sequence of the *alb*1 gene that encodes a polyketide synthase of the DHN-melanin pathway. Chromosomal DNA was isolated from WT, VCZ-R strains (n=5) and one clinical albino of *A. flavus*. From each isolate, *alb*1 with 3 highly conserved domains (HCDs) was amplified, sequenced and the deduced nucleotide/amino acid sequences were compared. In an attempt to evaluate the possible role of melanin pigmentation in VCZ-R, we proceeded with *alb*1 gene transformation and knock-out experiments, the results of which are outlined below.

PCR analysis of *alb*1 gene from a VCZ-R clinical albino strain of *A. flavus*

PCR-based amplification and subsequent characterization of the putative *alb*1 gene of *A. flavus* were performed per standard protocol, using primers 5'-atgcactagtactgagtttggaacaccc-3' and 5'-atgcgcggccgcgtacaattagtgcaagcc-3' (http://www.broadinstitute.org/annotation/genome/aspergillus_group/MultiHome.html. gene locusAFL2G_09923.2)

Gene transformation experiments (*alb*1)

Gene transformation experiments were then performed using the clinical strain VCZ-R AFL-R^{A1332V}*alb* and pBC-phleo as the cloning vector. The mutant *alb*1 (Δ*alb*1) was restricted using appropriate restriction enzymes and primers [forward primer 5`atgcactagtactgagtttggaacaccc (SpeI) and reverse prime 5`atgcgcggccgcgtacaattagtgcaagcc (NotI) [NEB Cutter V2.0 program, Ipswich, MA], cloned into pBC-phleo and used for transforming the wild-type *A. flavus* using phleomycin as the dominant selection marker.

Gene Knock-Out Experiments (*alb*1)

As a next step, we performed experiments using *alb*1 gene knock-out mutants, to confirm the role of *alb*1 gene in pigmentation Restriction analysis of *alb*1 gene and its flanking regions were carried out using NEB Cutter V2.0. DNA fragments specific to the flanking regions of *alb*1 gene were amplified by PCR, digested with restriction enzymes and inserted into unique sites of pPG28, which contains the *A. parasiticus pyr*G gene in a 2.7-kb BamHI-SalI fragment (obtained by courtesy from Dr PK Chang). The PCR primers used were (5`gcagcttataggagattcact gccgactggatcctctgtgt and 3` attgtcgacttccgatactatctacaatgcatgcaatactaccgatg). Mycelia for preparation of protoplasts were obtained from conidia of the CA14Δku70Δ*pyr*G *A. flavus* that is deficient in the non-homologous end joining site and auxotrophic to uracil, grown in PDB containing 0.5 mg uracil/ml. shaken at 150 rpm for 20 h at 30ºC. The disruption vectors were linearized prior to transformation to yield DNA ends that were identical to the targeting sites. Gene KO experiments were performed in a wild-type and VCR-R strains of *A. flavus*.

RESULTS

All *A. flavus* strains except control (MIC of 0.125mcg/ml) were resistant to VCZ (MIC of 8mcg/ml). Exposure of *A. flavus* to either TRO (4 mcg/ml) or TCZ (64 mcg/ml) resulted in albino colonies with white conidia. Interestingly, examination of the VCZ-R albino isolates of *A. flavus* demonstrated that despite loss of pigmentation, they continued to conidiate in a normal fashion. The extent of pigment inhibition was similar between TRO and TCZ. (Figure 1). Among the MIs tested, TRO showed significant activity (MIC 8 mcg/ml) against *A. flavus* whereas TCZ had comparatively weak activity (MIC 128 mcg/ml). The MFC (minimum fungicidal concentration) as determined using MTT-based spectrophotometric assay was 16mcg/ml and 256mcg/ml for TRO and TCZ respectively (Table 1).

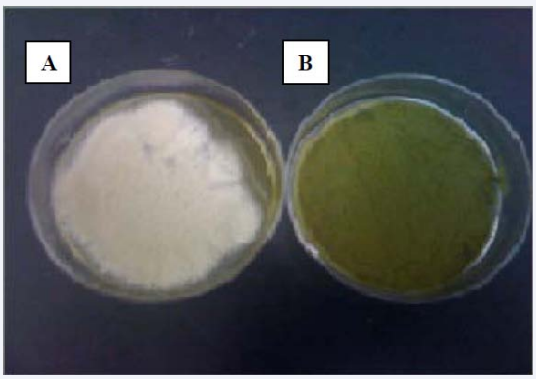

Figure 1 Inhibition of Melanin Pigmentation in *A. flavus.*
B. Green colony: Wild type *A. flavus* grown in SDA plate.
A. White colony: Wild type of *A. flavus* grown in SDA plate in the presence of fungal melanin inhibitors namely, tropolone (4 mcg/ml) or tricyclazole (64 mcg/ml); similar results for both melanin inhibitors

Table 1: *In vitro* Fungicidal Activity of Melanin Inhibitors with VCZ against VCZ-R *A. flavus.*

No.	*A. flavus* Isolate	MIC of VCZ	MIC of TRO	MIC of TCZ	FICI [VCZ (2 mcg/ml + TRO (2 mcg/ml)]	FICI [VCZ (2 mcg/ml) + TCZ (32 mcg/ml)]
1	AFL-S0188 (VCZ-S)	0.125	8	256	0.125	0.256
2	AFL-R100	8	8	256	0.25	0.125
3	AFL-R101	8	8	256	0.125	0.25
4	AFL-R102	4	8	256	0.5	0.25
5	AFL-R103	8	8	256	0.25	0.5
6	AFL-R104	8	8	256	0.5	0.25
7	AFL-R105	8	8	256	0.25	0.125
8	AFL-R106	4	8	256	1	0.25
9	AFL-R107	8	8	256	0.25	0.125
10	AFL-R108	8	8	256	0.125	0.25

MIC: Minimum inhibitory concentration (mcg/ml)
FICI: Fractional inhibitory concentration index
Synergy is defined as FICI <5

Effect of two-drug combinations

Combination of TRO or TCZ with VCZ reduced the MIC of all the drugs by 2 fold dilutions. The FICI of the different drug combinations at sub-MICs are shown in (Table 1). Sub-MIC of TRO (≤ 2 mcg/ml) or TCZ (≤ 32 mcg/ml) in combination with sub-MIC of VCZ (2 mcg/ml) demonstrated a FICI of < 0.5 thereby demonstrating a synergistic effect.

Kill curve experiments

Based on the results obtained from the FICI, kill curve experiments were set up using sub-MIC concentrations of VCZ in combination with either TRO or TCZ. Various concentrations of TCZ and TRO were tested in combination with 2mcg/ml of VCZ. Figures (1,2) show typical fungicidal activities of TRO, TCZ and VCZ either alone or in various combinations against VCZ-R *A. flavus* after 6, 18, 24 and 48 h of drug exposure. Sub-MIC values of VCZ (at 2 mcg/ml) in combination with sub-MIC of TRO (at 2 mcg/ml) demonstrated 95 - 99.9% killing (2-3 log drop in colony counts) of *Aspergillus* spores at 24 and 48h of exposure to drug whereas neither drug alone had significant fungicidal activity at sub-MIC values. On the other hand, sub- MIC of TCZ (at 32 mcg/ml) in combination with sub-MIC of VCZ (at 2mcg/ml) demonstrated ~ 90% killing at 24 and 48 hours.

PCR analysis of *alb*1 gene from a VCZ-R clinical albino strain of *A. flavus*

PCR-based amplification and subsequent sequence analysis of the *alb*1 gene of *A. flavus* revealed a protein having 1033 amino acid residues with 3 of the 5 highly conserved regions (β keto-acyl synthase domain, acyl transferase domain and the acyl carrier protein domains) reported in other fungi. A comparison of *alb*1 of WT *A. flavus* with that of NRRL 3357 (ATCC strain) revealed no strain dependent amino acid variations. However a comparison of the *alb*1 nucleotide sequence of wild type *A. flavus* with that of a unique clinical non-conidiating albino VCZ-R *A. flavus* strain revealed A1332V mutation in one of the highly conserved regions of *alb*1(AFL-R^{A1332V}*alb*). Interestingly, this clinical isolate exhibited complete loss of conidiation and grew exclusively by hyphal elongation. This clinical isolate lacked *cyp*51 mutations and was selected to assess the role of pigmentation in VCZ-R in *A. flavus*.

Gene transformation experiments (*alb*1)

A. flavus transform ants lost pigmentation and appeared as white colonies but continued to produce white conidia. Interestingly, these isolates demonstrated an increased susceptibility to VCZ. Our experiments demonstrated that A1332V mutation in *alb*1 gene [1] resulted in loss of pigmentation, [2] loss of pigmentation was associated with increased susceptibility to VCZ *in vitro* (Table 2).

Gene Knock-Out Experiments (*alb*1)

The *alb*1 knock-out (KO) strains demonstrated complete loss of pigmentation. Susceptibility testing using VCZ revealed a decrease in VCZ MIC by 2-fold dilution, from 0.125 to 0.0325mcg/ml in the wild type strain of *A. flavus*. Our experiments demonstrated that *alb*1 gene KO [1] resulted in loss of pigmentation, [2] loss of pigmentation alone (with preserved conidiation) was associated with hyper-susceptibility to VCZ (Table 2). The clinical albino strain however had loss of both pigmentation and conidiation and demonstrated resistance to VCZ, in the absence of *cyp*51A alterations.

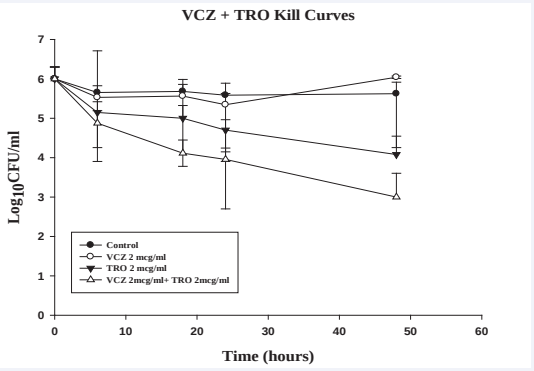

Figure 2 VCZ + TRO Kill Curves.

Table 2: Role of pigmentation and/or conidiation in VCZ-susceptibility in *A. flavus*.

A. flavus Strains	*alb*1	Pigmentation	Conidiation	VCZ-MIC
Wild-type AFLS0188	Normal	Normal	Normal	0.125 mcg/ml
Lab-selected VCZ-R *alb*1KO	Knock-out	Absent	Decreased by 50%	↓↓from 8 to 2 mcg/ml
Wild type *alb*1^{A1332V} transform-ant	*alb*1^{A1332V}a	Absent	Decreased by 50%	↓↓from 0.125 to 0.03 mcg/ml
Wild type *alb*1KO	Knock-out	Absent	Normal	↓↓from 0.125 to 0.03 mcg/ml
Clinical VCZ-R Albino Aconidial Strain (AFLR)	*alb*1^{A1332V}b	Absent	Absent	4 mcg/ml Resistant

*alb*1: melanin pigmentation gene
MIC: Minimum inhibitory concentration
KO: Knock-out
*alb*1^{A1332V}a: Loss of pigmentation only
*alb*1^{A1332V}b: Loss of pigmentation PLUS loss of conidiation

DISCUSSION

Although *A. flavus* ranks second to *A. fumigatus* in the USA, it is the most common pathogen implicated in a majority of cases of IA in several arid regions around the world. *A. flavus* is a species with several unique characteristics. It is the only mold that spans the agricultural, plant, insect and human worlds [25-39]. It is considered to be more virulent and less susceptible to triazoles, although literature pertaining to azole resistance in *A. flavus* is scarce.

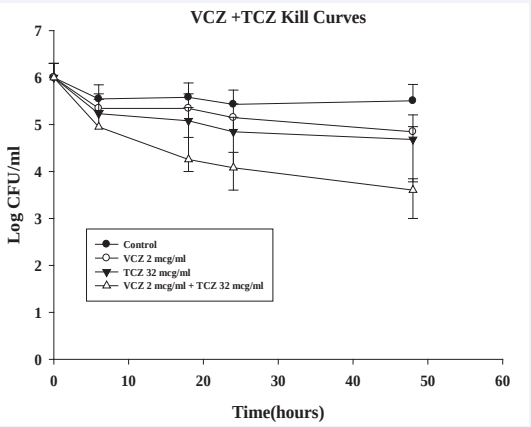

Figure 3 VCZ + TCZ Kill Curves.

Some earlier reports have demonstrated a novel effect of voriconazole on conidiation in *Aspergillus* species including *A. fumigatus*, *A. flavus* and *A. niger* [40]. Our experiments demonstrated that A1332V mutation in a HCD of *alb*1 gene or *alb*1 KO [1] led to loss of pigmentation and decreased conidiation [2] loss of pigmentation by itself did not result in VCZ-R [3] loss of conidiation (not loss pigmentation) likely contributed to VCZ-R in the clinical VCZ-R of *A. flavus*, which necessitates further investigation of the conidiation pathway in *A. flavus*. Hence we hypothesize that loss of conidiation may have contributed to VCZ-R in the albino isolate. Melanin inhibition in *A. flavus* by TRO and TCZ indicates the contribution of both DOPA and DHN-melanin pathways for melanin production in *A. flavus.* Tropolone and tricyclazole had good antifungal activity against VCZ-S and VCZ-R *A. flavus* albeit at high concentrations. Combination of sub-MIC of TRO or TCZ with sub-MIC of VCZ resulted in a 2-fold drop in MIC of all the drugs tested against VCZ-R isolates of *A. flavus.* Synergistic activity was observed with the combination of VCZ with TRO or TCZ.

Triazoles are known to exert their antifungal activity via competitive inhibition of the natural substrate cytochrome P450-dependent 14α-lanosterol demethylase ($P450_{14DM}$). Exposure of *A. flavus* strains to As a first step to evaluate the role of VCZ on the well described melanin pathway, the dominant gene of the melanin pathway namely *alb*1 was studied using gene transformation and KO experiments. Our *alb*1 transform ants and gene KO mutants revealed loss of pigmentation, retained conidiation and increased susceptibility to VCZ. Our observations suggest that [1] pigmentation and conidiation pathways are closely related [2] inhibition of melanin synthesis is associated with an increased susceptibility to VCZ [3] loss of conidiation and a switch to exclusive vegetative growth by hyphal elongation is associated with VCZ-R.

One theoretical explanation for the observed synergistic activity of VCZ with MIs is that melanin inhibition alters cell wall integrity facilitating the influx of a higher than normal concentration of VCZ to target the ergosterol pathway of the fungal cell membrane [41]. The exact nature of inhibition of the conidiation pathway by VCZ is purely speculative at this time. Observations from our study suggest a strong association between loss of conidiation and VCZ-R in *A. flavus.* The conidiation cascade has recently been shown to be regulated by a G-protein/cyclic AMP/Protein kinase/Signaling pathway and needs further evaluation. The asexual sporulation cycle in *Aspergillus* species is a highly genetically regulated process involving several genes and could be inhibited specifically by interfering with one or more steps involved in the asexual reproductive phase [42-44]. Emerging data from studies involving the conidiation pathway in *A. nidulans* have suggested that the secondary metabolites (? Melanin) are essential for growth and development of *Aspergillus* species [43,44]. The significance of a G-protein/cyclic AMP / protein kinase signaling pathway and its crucial role in conidiation has also been described [45]. A change in the gene expression profile of critical genes involved in this pathway could likely be associated with changes in triazole susceptibility and needs to be further explored. Inhibition of genes involved in this pathway leads to a switch from asexual reproduction by conidiation to loss of conidiation and pure vegetative growth by hyphal elongation [46]. Hyphal mats of Aspergillus are known to be relatively more resistant to antifungal killing compared to conidia.

Our experiments have several limitations [1] melanin inhibition by tropolone suggests likely involvement of the DOPA pathway that was not evaluated as it is a non-dominant pathway. We focused on the DHN-melanin pathway as it had been well established in various *Aspergillus* species [2] FICI is not a perfect method to assess for synergy as results cannot be directly extrapolated in *vivo* given the different pharmacokinetics in play [3] a direct relationship between pigmentation and conidiation pathway could not be determined [4] small sample number and [5] some were laboratory-selected VCZ-R isolates.

In conclusion, observations from our study suggest a novel association of melanin pigmentation and/or conidiation pathway and triazole susceptibility in *A. flavus*. It is also the first study to investigate the antifungal activity of melanin inhibitors in combination with triazole against *A. flavus.* More studies are needed to explore these pathways in order to understand the reasons for the observed synergistic interaction. Also, the role of conidiation cascade, if any in triazole resistance, needs further investigation. Research in this field will help understand the possible non-*cyp*51A related molecular mechanisms of triazole resistance and open avenues to circumvent antifungal drug resistance and aid in the development of novel therapeutic strategies for the treatment of invasive aspergillosis.

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