F1-1382. In *Vitro* Synergistic Fungicidal Activity of Tricyclazole (TCZ) and Tropolone (TRO) in Combination with Voriconazole (VCZ) against VCZ-Resistant (R) *Aspergillus flavus.*

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ABSTRACT

Background: Tropolone and Tricyclazole are known melanin inhibitors (MIs) that inhibit melanin via DOPA (di-hydroxyphenylalanine) or di-hydroxynaphthalene melanin (DHN-M) pathways respectively. **AIM:** (i) To investigate the involvement of these pathways in melanin pigmentation in *A. flavus* and (ii) to evaluate the in *vitro* fungicidal activity of the combination of melanin inhibitors and VCZ against VCZ-R *A. flavus*. **Methods:** Fresh conidial suspensions were prepared from 6-day old *A. flavus* cultures (n=10; 1 wild type and 9 VCZ-R *A. flavus* with MIC > 4 mcg/ml) grown on SD agar +/-TCZ and TRO (at 8 mcg/ml) to evaluate for melanin inhibition. MIC, FICI (fractional inhibitory concentration index), MTT and time kill assays (at 0, 6, 18, 24 and 48 hrs) 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 and confirmed using MTT assay. **Results:** *A. flavus* lost its pigmentation in the presence of 8 mcg/ml of TCZ or TRO; TCZ at 256 mcg/ml) and TRO at 8 mcg/ml) demonstrated intrinsic antifungal activity against WT and VCZ-R *A. flavus*. Sub-MICs of MIs plus VCZ demonstrated synergism against wild type (WT) and VCZ-R *A. flavus* at 24h and 48h with FICI < 0.5. Subsequent MTT-based spectrophotometry and time kill assays showed 90-99 % killing of *Aspergillus* spores at 48 hrs. Neither VCZ nor MIs alone showed fungicidal activity at sub-MIC against VCZ-R *A. flavus*. **Conclusions:** Both TCZ and TRO (MIs) have reasonable intrinsic antifungal activity against *A. flavus* and demonstrate good synergistic antifungal activity when used in combination with VCZ against VCZ-R *A. flavus* and need further investigation.

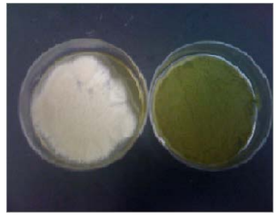
INTRODUCTION

Invasive aspergillosis is a life threatening infection in immunocompromised patients especially following hematopoietic stem cell transplantation and graft vs host disease. Despite the availability of various antifungal agents, the mortality rate approaches 80-90% and clearly new therapeutic approaches are needed. Combination therapy is one approach that can be used to improve the efficacy of antimicrobial therapy. Most combination studies focus on agents that have complementary targets within the fungal cells (polyenes plus triazoles or echinocandins or antifungals plus immune factors, neutrophils etc.). The potential advantages of using combination therapy include broad spectrum of activity, greater potency than the individual drugs used in monotherapy, reduction in the organisms and a good safety profile if lower drug concentrations could be used.

A. flavus conidia are the first and foremost structures encountered by the human host, are 3-5 microns in diameter, are hydrophobic with yellowish green pigmentation. At the initial phase of fungal-host interaction, the conidial surface components play an integral role in evasion of the innate and acquired immune defense mechanisms. Fungal pigments, most importantly melanin, have been shown to contribute to fungal invasion, evasion and virulence in several pathogenic fungi including *Aspergillus species*, *Cryptococcus* and *Wangiella dermatitidis.*

Pigment inhibition by tropolone (via DOPA-melanin pathway) and tricyclazole (via DHN-melanin pathway), has confirmed the presence of both these pathways in *A. flavus* (figure). Previous in *vivo* studies have suggested the possible role of melanin in virulence and pathogenesis of *A. fumigatus*. However none of the studies have evaluated the in *vitro* efficacy of MIs against *A. flavus*. In this study we evaluated the in *vitro* efficacy of TCZ and TRO alone and in combination with VCZ against VCZ-susceptible and VCZ-R *A. flavus*.

Inhibition of Pigmentation by MIs in *A. flavus*



MATERIALS

Antifungal drugs:
Voriconazole was obtained from Pfizer Pharmaceuticals (New York, NY,USA). VCZ, TCZ and TRO were dissolved in dimethylsulphoxide to make a stock solution of 1g/L and then stored as 0.25ml aliquots at –20ºC.. Frozen stocks of the antifungal agents were thawed at room temperature and used within 24h. Melanin inhibitors used in this study were obtained from Sigma-Aldrich, USA as pure powders.
Clinical isolates:
The 9 VCZ-R isolates of *A.flavus* used in this study were selected in the laboratory by a 2-step selection process by exposure to VCZ (range 0.5 to 4 mcg/ml).
The original cultures were subcultured in Sabouraud dextrose agar to check for purity and viability. Working cultures were maintained on Sabouraud dextrose agar slants at 4ºC. For long term preservation of the cultures, conidial suspensions were prepared in glycerol 25% v/v and stored at –80ºC.
MIC determination:
Conidial suspensions from 6 day old *A.flavus* cultures were prepared, standardized by hemocytometry and used as inocula (2 X 10 ^{4}) for susceptibility testing. MICs of voriconazole, tropolone and tricyclazole for the 10 isolates of *A.flavus* were determined by the CLSI M38-A2 methodology. MIC was defined as the concentration of drug that resulted in 100% visual inhibition of growth. Drug concentrations ranging from 0.125 to 16 mcg/ml (voriconazole) or 1 mcg/ml to 512 mcg/ml (TCZ and TRO) were used for MIC determinations. Each MIC determination was repeated once.

METHODS

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 . 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) and antagonistic (FICI>4).

Kill-curve studies to measure the fungicidal activity:
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 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 was removed and diluted to obtain 10-10^3 dilutions and 0.1ml aliquots were spread on SDA plates and incubated at 35° C for 24h 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 antifungal agents.

RESULTS

Among the MIs tested, TRO showed significant activity (MIC: 8 mcg/ml) against *A. flavus* whereas TCZ had comparatively weak activity (MIC: 256 mcg/ml). The MFC (minimum fungicidal concentration was 16 mcg/ml and 256 mcg/ml for TRO and TCZ respectively. All *A. flavus* strains except control (MIC of 0.25mcg/ml) were resistant to VCZ (MIC of 8 mcg/ml) Table 1.

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 an average 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 2 mcg/ml of VCZ. Fig 1 and 2 show typical fungicidal activities of several drugs either alone or in combinations against *A.flavus* after 6, 18, 24 and 48h 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 2 mcg/ml) demonstrated ~ 90% killing at 24 and 48 hours. Thus we demonstrated that melanin inhibition resulted in significant antifungal activity against *A. flavus* with the best activity displayed by TRO at significantly lower concentrations as compared to TCZ. Our results also demonstrate that both pathways are involved in melanin synthesis in *A. flavus*. However, the reasons for the observed difference in antifungal activity between TRO and TCZ is unclear at this time.

Figure 1:

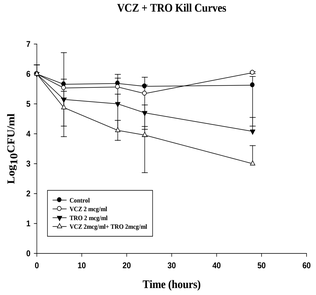


Figure 2:

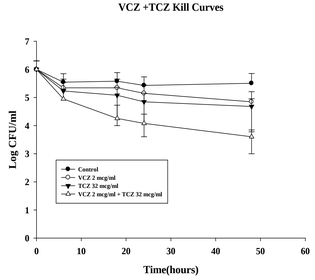


Table 1:

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

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

DISCUSSION

- Melanin inhibition in *A. flavus* by tropolone (inhibition of DOPA-melanin pathway) and tricyclazole (inhibitor of reductases in DHN-melanin pathway) indicates the contribution of both pathways for melanin production in *A. flavus.*
- Tropolone and tricyclazole exhibit reasonable 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 but was less pronounced with TCZ the reason for which is unclear although it may be plausible that the DOPA pathway using tyrosinase may be the dominant pathway in *A. flavus.*
- The theoretical explanation for the observed synergistic activity is likely due altered cell wall integrity by melanin inhibitors plus some activity of VCZ on fungal cell membrane. Similar results were seen with VCZ-S and VCZ-R *A. flavus.*
- In conclusion, this is the first study in which the activity of fungal melanin inhibitors in combination with triazole was investigated in *A. flavus.* Further studies are needed to understand the mechanisms underlying the observed synergistic interaction and are warranted in order to develop new therapeutic strategies for the treatment of invasive aspergillosis.