Interaction Between Amorolfine and Voriconazole Against Fusarium species

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Abstract Fusarium species represent a range of fungal pathogens capable of causing diverse mycotic diseases. Relative to antibacterial drugs, few effective antifungal agents have been developed to date, and all are subject to significant limitations. As such, there is an urgent need to design novel antifungal treatments for infections caused by Fusarium spp. Herein, 15 clinical isolates, including 5 Fusarium oxysporum and 10 Fusarium solani strains, were analyzed to explore the relative inhibitory effects of different combinations of amorolfine (AMO) and voriconazole (VOR) on the growth of these fungal pathogens. These analyses were conducted by measuring minimal inhibitory concentration (MIC) values for these antifungal agents in a broth microdilution assay and by using an in vivo model of Fusarium-infected Galleria mellonella. These experiments revealed that in isolation, AMO and VOR exhibited MIC values ranging from 4 to 16 µg/mL and 2 to 8 µg/mL, respectively. However, these effective MIC values fell to 1–2 µg/mL and 0.5–2 µg/mL, respectively, when AMO and VOR were administered in combination with one another, exhibiting synergistic activity against 73.3% of analyzed Fusarium strains. Subsequent in vivo analyses conducted using the G. mellonella model further confirmed that combination VOR + AMO treatment was associated with significantly improved larval survival following Fusarium spp. infection. Together, these results serve as the first published evidence demonstrating that VOR and AMO exhibit synergistic activity against infections caused by Fusarium spp., indicating that they may represent an effective approach to antifungal disease treatment.

Keywords Fusarium species · Amorolfine · Voriconazole · Antifungal · Synergistic

Introduction

Fusarium species are fungi that are present throughout a diverse range of environmental conditions [1], which can induce a wide array of superficial, locally invasive or disseminated infections in humans and other species [1–20]. The inhalation of Fusarium conidia can lead to the development of an infectious condition known as fusariosis, which arises when these conidia are able to germinate within an appropriate physiological niche and to subsequently form filamentous structures capable of invading local tissues [20]. Tamura et al. determined that Fusarium spp. were resistant to in vitro treatment with most tested antifungal agents [21]. At present, invasive fusariosis and other Fusarium infections are most
commonly treated using voriconazole (VOR) or liposomal amphotericin B [22–24]. Even with such treatments, however, many patients experience poor outcomes linked to a failure to reestablish normal immunological functionality, with neutropenia being closely linked to a poor prognosis [25]. Individuals undergoing treatment with VOR or liposomal amphotericin B are also at an elevated risk of adverse reactions including a decline in normal renal function. In contrast to antibacterial drugs, which are relatively common, effective antifungal agents remain rare and are associated with significant limitations. There is thus an urgent need for the development of novel antifungals and combinatorial therapeutic regimens capable of reliably treating severe fungal infections.

Amorolfine (AMO) is a morpholine derivative and a broad-spectrum antifungal drug that has been used to treat a range of fungal infections in humans caused by opportunistic pathogenic fungi, yeasts and dermatophytes [26]. In prior reports, a combination of AMO and itraconazole treatment was shown to exhibit synergistic activity against most tested dermatophytes [21].

Herein, we employed a checkerboard microdilution approach to assess the antifungal activity of a combination of AMO and VOR in vitro against clinical Fusarium isolates. We then expanded these assays by assessing the in vivo effects of combination AMO + VOR treatment on Galleria mellonella larvae infected with Fusarium spp. in order to monitor for treatment-related changes in larval survival.

Materials and Methods

Fungal Strains

In total, 15 clinical Fusarium isolates were utilized (Table 1), including 10 Fusarium solani complex strains and 5 Fusarium oxysporum complex strains. Microscopic examination, together with ITS and TEF sequencing, was used to confirm the identities of these Fusarium spp. in a previously published study [27].

Broth Microdilution Assay

The effects of AMO, VOR, or a combination of both drugs were tested using all 15 Fusarium isolates detailed above, with Aspergillus flavus (ATCC 204,304) serving as a control. Fusarium spp. susceptibility assays were conducted as per Clinical and Laboratory Standards Institute document M38-A2 [28]. First, the individual minimal inhibitory concentration (MIC) values for VOR and AMO were established, after which combination assays were performed. In checkerboard assays [28], dimethyl sulfoxide (DMSO) was utilized to prepare VOR and AMO stocks (3200 µg/ml) from which working concentrations were prepared in RPMI-1640 via serial dilution for both VOR (0.25–16 µg/mL; Shanghai Selleck Co., Ltd., China) and AMO (0.5–32 µg/mL; Shanghai Selleck Co., Ltd., China).

Fractional inhibitory concentration index (FICI) values were used to assess interactions between VOR and AMO and were calculated as follows: FICI = (Ac/Aa) + (Bc/Ba), where Ac and Bc correspond to the MIC values for these drugs in combination, while Aa and Bb correspond to the MIC values for these drugs when used as single agents. FICI values ≤ 0.5 correspond to synergistic activity, whereas a FICI from 0.5 to 4 is indicative of a lack of any interaction, and a FICI value > 4 indicates an antagonistic interaction [29]. All analyses were performed in triplicate.

Galleria mellonella Assay

Galleria mellonella larvae were utilized as discussed previously [30–32] and were separated into six treatment groups: untreated (uninfected larvae not subjected to any treatments), saline (uninfected larvae injected with 10 µL of saline), conidial (F. solani complex-infected larvae), VOR (100 µg/mL)-treated (F. solani complex-infected larvae treated with VOR), AMO (100 µg/mL)-treated (F. solani complex-infected larvae treated with AMO), and VOR (100 µg/mL) + AMO (100 µg/mL)-treated (F. solani complex-infected larvae treated with VOR and AMO) groups. A total of 20 larvae (0.3–0.4 g in weight) were included per experimental group, with experiments being repeated three times. A single Fusarium solani complex isolate (jzfs 1) was utilized for all in vivo analyses. Briefly, Fusarium jzfs1 conidia were collected following a 1-week culture grown on PDA at 28 °C for 1 week, with the agar surface being washed with PBS following by hemocytometer-mediated conidial counting (10^7 CFU/ml). Next, 10 µL of a conidial suspension was injected into G. mellonella larvae in all groups other than the untreated and saline
control groups, after which larvae were incubated at 37 °C for 2 h, followed by treatment with appropriate antifungal drugs (5 μL). Larvae were then incubated at 37 °C and monitored once per day for 6 days to assess survival outcomes.

**Histological Analyses**

To examine *G. mellonella* tissues for the presence of *Fusarium* isolates, larvae from the different treatment groups were collected on day 3 post-infection. Larvae were then prepared as discussed previously [27], first being fixed, dehydrated with an ethanol gradient series (70, 80, 90, 96, and 100%), sliced to generate ultrathin sections, stained with hematoxylin and eosin (H&E), and examined with an FSX100 fluorescence microscope (Olympus, Tokyo, Japan) at 10× and 40×.

**Statistical Analysis**

GraphPad Prism 5.0 was utilized for figure preparation and statistical analyses. *G. mellonella* survival outcomes were assessed with Kaplan–Meier curves and log-rank (Mantel-Cox) tests, with *P* < 0.05 as the significance threshold.

### Results

Assessment of the antifungal activity of AMO and VOR against *Fusarium* spp.

For all tested *Fusarium* isolates, MIC values for AMO and VOR ranged from 4 to 16 μg/mL and 2 to 8 μg/mL, respectively (Table 1). Combinations of VOR and AMO exhibited synergistic activity against 11 *Fusarium* strains (73.3%), with lower MIC values ranging from 1 to 2 μg/ml for AMO and 0.5 to 2 μg/mL for VOR. FICI values further confirmed these in vitro results, with values for most *F. solani* complex (Jzfs1, Jzfs2, Jzfs3, Jzfs4, Jzfs6, Jzfs7, Jzfs8, Jzfs10) and *F. oxysporum* (Jzfo2, Jzfo3, Jzfo5) strains being ≤ 0.5 (Table 1). Even so, FICI values were > 0.5 for certain *Fusarium* strains, including the Jzfs5, Jzfs9, Jzfo1, and Jzfo4 strains, indicating a lack of synergistic activity against these strains.

### Efficacy of VOR and AMO in *Fusarium*-infected *G. mellonella*

Next, we explored the in vivo antifungal efficacy of VOR and AMO in combination with one another or in isolation by using a model system consisting of *G.*

### Table 1 MIC and FICI results with combination of AMO and VOR against *Fusarium* spp

| Strains | Origin     | MICs (μg/ml) | AMO | VOR | AMO/VOR | FICI | IN  |
|---------|------------|--------------|-----|-----|---------|------|-----|
|         |            |              |     |     |         |      |     |
| *F. solani* |            |              |     |     |         |      |     |
| Jzfs1   | Skin       | 4            | 4   | 1/0.5 | 0.375 | SYN  |     |
| Jzfs2   | Skin       | 8            | 4   | 1/0.5 | 0.25  | SYN  |     |
| Jzfs3   | Cornea     | 4            | 2   | 1/0.5 | 0.5   | SYN  |     |
| Jzfs4   | Cornea     | 8            | 4   | 2/1   | 0.5   | SYN  |     |
| Jzfs5   | Skin       | 4            | 2   | 2/0.5 | 0.75  | NI   |     |
| Jzfs6   | Cornea     | 16           | 8   | 1/1   | 0.1875| SYN  |     |
| Jzfs7   | Skin       | 8            | 4   | 1/0.5 | 0.25  | SYN  |     |
| Jzfs8   | Auditory canal | 8   | 2   | 1/0.5 | 0.375 | SYN  |     |
| Jzfs9   | Auditory canal | 8   | 2   | 1/1   | 0.625 | NI   |     |
| Jzfs10  | Nail       | 16           | 8   | 2/2   | 0.375 | SYN  |     |
| *F. oxysporum* |            |              |     |     |         |      |     |
| Jzfo1   | Cornea     | 8            | 2   | 1/1   | 0.625 | NI   |     |
| Jzfo2   | Nail       | 16           | 4   | 1/0.5 | 0.1875| SYN  |     |
| Jzfo3   | Skin       | 8            | 4   | 2/0.5 | 0.375 | SYN  |     |
| Jzfo4   | Skin       | 8            | 2   | 2/1   | 0.75  | NI   |     |
| Jzfo5   | Cornea     | 8            | 2   | 1/0.5 | 0.375 | SYN  |     |

**Syn**, synergism (a FICI of ≤ 0.5); **NI**, no interaction (indifference, a FICI of > 0.5 to ≤ 4); **AMO**, amorolfine; **VOR**, voriconazole; **FICI**, fractional inhibitory concentration index; **IN**, interaction; **MICs**, minimal inhibitory concentrations


*mellonella* larvae infected with the jzfs1 *Fusarium* isolate. On day 2 postinfection, VOR + AMO treatment was associated with higher survival rates (68%) relative to either monotherapy (VOR, 51%; AMO, 43%) or to infected but untreated larvae (28%). On day 4 post-infection, survival rates in larvae treated with VOR + AMO were threefold higher than those in the untreated conidia-infected group. On day 6, the survival rates of larvae in the AMO, VOR, and AMO + VOR groups were 12%, 15%, and 30%, respectively, confirming that AMO + VOR treatment significantly improved larval survival as compared to that observed in the AMO group (P = 0.0013), VOR group (P = 0.0318), and conidial group (P < 0.0001) (Fig. 1). Together, these data indicate that AMO and VOR exhibit synergistic activity when used to treat *G. mellonella* infected with *F. solani* complex.

**Histopathological Analyses**

On day 3 post-infection, histopathological analyses of *F. solani*-infected *G. mellonella* larvae that had or had not been treated with AMO, VOR, or AMO + VOR were conducted. High numbers of fungi were evident in stained larval tissue sections, with evidence of extensive tissue damage in larvae from the conidial group and the presence of high levels of spores and mycelia (Fig. 2a). A decrease in levels of tissue damage and fungal abundance was evident in the combination VOR + AMO treatment group (Fig. 2d) relative to the VOR group (Fig. 2c), the AMO group (Fig. 2b), and the conidial group. The VOR-treated larvae also exhibited slight reductions in numbers of spores and mycelia as compared to the AMO and conidial groups.

**Discussion**

In most cases, fusariosis can be effectively treated with VOR or amphotericin B [20, 33], but there remain many barriers to the reliability of these treatments, which can also be relatively expensive. The application of novel compounds or other drugs in combination with azoles may be an effective means of treating fungal infections, improving overall antifungal efficacy while reducing treatment-related adverse events. In prior reports, licofelone was shown to synergize with fluconazole when treating fluconazole-resistant *C. albicans* conidia [30]. Stempel et al. [34] further found that a combination of VOR with terbinafine was associated with enhanced efficacy when used to treat fusariosis, while pyrvinium pamoate was shown to synergize with azole drugs in the treatment of *Exophiala* dermatitidis [35]. Kathrin et al. [36] also determined that combinations of VOR and micafungin synergized against *F. solani* complex in vitro. As a broad-spectrum antifungal drug with activity against opportunistic fungi, yeast, and dermatophytes [26], AMO represents a promising tool with previous documented synergistic efficacy against many dermatophytes [21].

![Fig. 1 Galleria mellonella survival rates under different treatment conditions. Curves correspond to untreated (larvae free of *F. solani* infection or other treatments), saline (saline-injected larvae), conidial (*F. solani* complex-infected larvae), AMO (*F. solani* complex-infected larvae treated with AMO), VOR (*F. solani* complex-infected larvae treated with VOR), and AMO + VOR (*F. solani* complex-infected larvae treated with both AMO and VOR) groups. AMO: amorolfine; VOR: voriconazole. Experiments were repeated thrice, with 20 larvae per group (0.3–0.4 g in weight). *P < 0.05; **P < 0.01; ***P < 0.001](image-url)
Herein, we assessed the synergistic activity of VOR and AMO against Fusarium isolates by calculating the MIC values for these drugs together and in isolation, confirming that such synergy was evident for the majority of tested strains. In contrast, no synergy was observed for 2/10 *F. solani* complex isolates (20%) and 2/5 *F. oxysporum* complex isolates (40%) (Table 1), possibly because our sample size was relatively limited or due to the different mechanisms whereby these drugs impact particular fungal strains. In future analyses, we will analyze larger numbers of *F. oxysporum* complex strains to better validate these findings. Importantly, we observed no antagonistic interactions between these two drugs.

*Galleria mellonella* larvae have recently been identified as an ideal in vivo model for the preclinical analysis of the antifungal activity of novel drugs, as these larvae exhibit immune responses similar to those of mammals without any of the concomitant ethical concerns, and they also offer advantages such as being inexpensive and easy to manipulate [30, 37, 38]. To further verify the interaction between AMO and VOR detected in our study, we tested the in vivo effect of this combination against one of the isolates of *F. solani* complex with *G. mellonella* model. We found that AMO-VOR combination treatment was associated with significant improvements in larval survival as compared to VOR or AMO treatment in isolation, confirming the synergistic benefits of these two drugs when used to treat infections caused by *Fusarium* spp.

VOR is an azole antifungal compound capable of disrupting ergosterol biosynthesis within the fungal cell membrane via inhibiting the activity of lanosterol 14α-demethylase [39–41]. This mechanism of action is distinct from that of AMO, which interferes with the Δ14-sterol reductase and Δ7–Δ8-sterol isomerase pathways within target fungal cells [42]. As such, AMO and VOR may be able to effectively synergize owing to their ability to target different aspects of ergosterol biosynthesis pathways. Muller et al. have also reported that AMO could compromise the integrity of mitochondria, nuclear membranes, cell walls, and other structures in fungal cells, eventually leading to their death [43], further explaining its ability to synergize with VOR.

Khan et al. previously found that the antifungal activity of AMO was limited to superficial fungal infections, and that it was not systemically active in murine model systems, likely owing to its rapid metabolic processing and/or robust tendency to bind to proteins in vivo [44, 45]. The 15 clinical *Fusarium* isolates utilized herein had been primarily derived from the skin, corneal tissues, auditory canals, and nails of infected patients. As such, combination treatment with AMO and VOR may only be viable when treating superficial fungal infections caused by *Fusarium* spp., such as fungal keratitis.

We additionally analyzed larval morphology via microscopy following infection and treatment (Fig. 2). These histological analyses indicated that combination VOR + AMO treatment was associated with a reduction in *Fusarium* levels and corresponding tissue damage in *G. mellonella* larvae relative to corresponding controls, confirming the synergistic activity of VOR and AMO against *Fusarium* spp. infections.

Together, the results of this study indicate that AMO can effectively synergize with VOR in the treatment of infections caused by *Fusarium* spp., suggesting that combining these two drugs may be a viable antifungal approach to alleviating superficial fungal infections caused by *Fusarium* spp., such as fungal keratitis. However, future comprehensive analyses will be necessary to establish the mechanistic basis for the synergistic antifungal activity of VOR and AMO in order to guide the design of novel therapeutic regimens.

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**Authors’ contributions** PL and QL conceived and designed the study; IS performed sample collection and fungal culture; SJ and PZ analyzed the data; PL and QL wrote the manuscript. All authors read and approved the final manuscript.
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All data generated or analyzed during this study are included in this published article. The data used or analyzed during the current study are available from the corresponding author on reasonable request.

All authors declare that they have no conflicts of interest.

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