AAC Accepts, published online ahead of print on 4 March 2013
Antimicrob. Agents Chemother. doi:10.1128/AAC.02457-12

Antimicrobial Agents and Chemotherapy

Title: Farnesol against *Coccidioides posadasii*: its effect on growth, ergosterol biosynthesis and cell permeability

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Running title: Farnesol against *Coccidioides posadasii*

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Abstract

Coccidioidomycosis is a systemic mycosis caused by the dimorphic fungi *Coccidioides* spp. The treatment for chronic and/or disseminated coccidioidomycosis can be prolonged and complicated. Therefore, the search for new drugs is necessary. Farnesol is a precursor in the sterol biosynthesis pathway that has been shown to present antifungal activity. Thus, the objective of this study was to evaluate the *in vitro* antifungal activity of farnesol alone and in combination with antifungal agents against clinical and environmental strains of *Coccidioides posadasii*, as well as to determine their effect on the synthesis of ergosterol and on cell permeability. This study employed the broth macrodilution method to determine the minimum inhibitory concentration (MIC) of farnesol against 18 strains of *C. posadasii*. Quantification of ergosterol was performed with ten strains of *C. posadasii*, after the exposure to sub-inhibitory concentrations of farnesol. Finally, the activity of farnesol was evaluated at the presence of osmotic stress, induced by the addition of NaCl to the culture medium, during the susceptibility tests. The results showed that farnesol exhibited low MICs (ranging from 0,00171- 0,01369 mg/L) against all tested strains. The combination of farnesol with the antifungals showed synergistic effects (FICI≤0.5). As for ergosterol quantification, it was observed that exposure to sub-inhibitory concentrations of farnesol decreased the amount of ergosterol extracted from the fungal cells. Furthermore, farnesol also showed lower MIC values when the strains were subjected to osmotic stress, indicating the action of this compound on the fungal membrane. Thus, due to the high *in vitro* antifungal activity, this work brings perspectives for the performance of *in vivo* studies to further elucidate the effects of farnesol on the host cells.

Introduction

Coccidioidomycosis is a systemic mycosis caused by the dimorphic and geophilic fungi *Coccidioides immitis* and *Coccidioides posadasii* (1), which usually is a benign infection with spontaneous resolution. However, a small proportion of infected individuals develop the progressive form, which is potentially lethal and can affect not only the lungs, but other organs, through hematogenous dissemination (2).

The treatments for chronic and disseminated coccidioidomycosis can be prolonged and often complicated (3). Thus, there is a need for studies that seek new therapeutic options to treat coccidioidomycosis, since amphotericin B, considered the drug of choice for the treatment of the disease, is a potentially nephrotoxic drug (4) and only 50-60% of these patients are responsive to treatment with itraconazole and fluconazole (3).

Farnesol is a sesquiterpene that acts as a precursor in the biosynthesis of sterols and isoprenoids in *Candida albicans* (5, 6). Studies have shown that it acts as a quorum-sensing molecule and is involved in the inhibition of filamentation (5, 6) and the formation of biofilms (7, 8). More recently, it has been demonstrated that this compound also has an important role in the resistance to oxidative stress (9). However, farnesol has a cytotoxic effect on *C. albicans* at certain concentrations and under some environmental conditions (10), as well as against other microorganisms, inducing apoptosis (11).

Some studies have confirmed the inhibitory effect of farnesol on the growth of microorganisms (12-16). It was shown that farnesol can act as an antifungal agent against the dimorphic fungus *Paracoccidioides brasiliensis* (16). More recently, our

group demonstrated its inhibitory activity against *Cryptococcus* spp. (17) and *Candida* spp. (18).

Thus, the objective of this study was to evaluate the *in vitro* antifungal activity of farnesol alone and in combination with antifungal agents against clinical and environmental strains of *Coccidioides posadasii,* as well as to determine its effect on the synthesis of ergosterol and on cell permeability.

Materials and Methods

Fungal culture

This study included 18 strains (15 clinical and 3 environmental) of *Coccidioides posadasii* from Northeastern Brazil. All strains belong to the fungal collection of the Specialized Medical Mycology Center (CEMM, Federal University of Ceará, Brazil). The procedures for identification of the fungi included classic mycological analysis, as described by Brilhante et al. (2008) (19), and a PCR assay (20). All procedures were performed in a class II biological safety cabinet in a biosafety level 3 laboratory.

Inoculum preparation for antifungal susceptibility testing

C. posadasii strains were grown on potato agar and incubated for 7 days at room temperature (25 to 28°C). To prepare the inoculum, 2 mL of sterile saline were added to each culture and, with the aid of a microbiological loop, the surface of the mycelium was scraped. The suspensions were transferred to sterile tubes and allowed to stand for 5 minutes. The supernatant was read in a spectrophotometer at 530 nm and its transmittance was set to 95%. The suspensions containing arthroconidia and hyphal

fragments were diluted to 1:10 with RPMI 1640 and buffered with MOPS 0.156M to pH 7.0, to obtain inocula of approximately 1 x 10^3 to 5 x 10^3 CFU/mL^{-1} (21).

Antimicrobial agents and *In vitro* susceptibility testing

The solutions tested were prepared at the time of use from the commercial solution of 95% farnesol (mixture of isomers; Sigma-Aldrich), using 30% DMSO as a solvent. For the susceptibility assay, farnesol was further diluted with RPMI 1640 with L-glutamine, buffered to pH 7.0 with 0.165M MOPS, until reaching the concentration range of 0.00020-0.0548 mg/L.

After determining the minimum inhibitory concentration (MIC) of farnesol and the antifungal agents alone, we tested combinations of farnesol with amphotericin B, itraconazole, voriconazole and caspofungin. The combinations were tested in the following concentration range: 0.00000667-0.0137 mg/L for farnesol, 0.0039-0.125 mg/L for amphotericin B, 0.0156-0.5 mg/L for itraconazole, 0.0078-0.25 mg/L for voriconazole and 2-32 mg/L for caspofungin. The initial concentrations of the antifungals and farnesol represented the MICs found for these compounds individually against each tested strain.

The susceptibility of *C. posadasii* strains to farnesol and antifungals alone and in combination was determined through the broth macrodilution method, according to the M38-A2 protocol standardized by the CLSI (22). The results obtained were visually read, after 48 hours of incubation at 35 °C. The MICs for farnesol (17), itraconazole, voriconazole and caspofungin alone or in combination were defined as the lowest concentration of drug capable of inhibiting 80% of fungal growth, when compared to the drug-free control tube (23). As for amphotericin B alone, the MIC was the lowest

concentration at which no fungal rowth was observed. For quality control of the antifungal susceptibility tests, *Candida parapsilosis* ATCC 22019 was included.

The interaction between the combined drugs was evaluated by calculating the fractional inhibitory concentration index (FICI), according to Johnson et al. (2004), where FICI values of ≥0.5 indicate synergism, 0.5> FICI <4.0 indicate indifferent interactions and FICI ≥4.0 indicate antagonism (24). The differences between the MICs of drugs individually and in combination were evaluated by Student's t test. The obtained FICI values for each drug combination were compared through Student's t test. P-values lower than 0.05 indicated statistically significant differences.

Extraction of ergosterol

Cellular ergosterol was extracted as described by Arthington-Skaggs (25), with some modifications. The extraction was performed after the exposure of ten strains (05-2-064; 05-2-066; 05-2-067; 05-2-068; 05-2-070; 01-6-091; 01-6-092; 01-6-101; 01-6-102; 01-6-103) of *C. posadasii* to sub-inhibitory concentrations of farnesol and itraconazole (control drug), through the macrodilution technique. Seven concentrations of the compounds were tested, ranging from 0.0000133 to 0,003469 mg/L for farnesol and from 0.00195 to 0.125 mg/L for itraconazole. The mycelial pellet for each concentration was exposed to 0.5 mL of KOH/EtOH (20%/60%) and incubated at 95 °C, for 1h, in a water bath. After that, 0.5 mL of hexane was added in order to isolate the sterols. The solutions were centrifuged for two minutes (10,000 xg). Then, the top layer of hexane was transferred to microtubes and added to 0.5 mL of hexane. Ergosterol quantification was performed in a spectrophotometer at λ = 295.10 nm and

compared to a predetermined standard curve. For positive control, ergosterol from the ten evaluated strains of *C. posadasii* grown in drug-free RPMI medium was quantified.

Inhibitory effect of farnesol in the presence of osmotic stress

The ability of farnesol to alter the permeability of the fungal memebrane was also evaluated by macrodilution. To induce osmotic stress, we used the method described by Coleman et al. (2010) with modifications, where the RPMI 1640 medium buffered to pH 7.0 was added to NaCl 0.175 M (26). The concentration of NaCl was previously determined through macrodilution in the range of 7 to 0.0021 M. Concentrations ≤ 0.175 M did not interfere with the growth of *C. posadasii*, when compared to the drug-free salt-free control. Lastly, sub-MIC concentrations of farnesol were tested. The results were visually read after 48h of incubation at 35 °C.

Results

MIC Farnesol alone and in combination with antifungal drugs

All strains of *C. posadasii* were inhibited by low farnesol concentrations, with MICs ranging from 0.00171 to 0.01369 mg/L (0.0078-0.0616 μM) and a geometric mean of 0.00634 mg/L (0.285 μM). For the antifungal drugs in clinical use, the MIC intervals found in mg/L were 0.0625-0.125 for amphotericin B, 0.125-0.5 for itraconazole, 0.125-0.25 for voriconazole and 16-32 for caspofungin. In addition, all drug combinations tested were able to inhibit growth of *C. posadasii* at lower doses and a significant reduction was found for all tested drugs (caspofungin $P<0.0001$, itraconazole $P=0.0016$, amphotericin B $P<0.0001$ and voriconazole $P=0.0002$), with a statistically significant synergistic effect for the combinations of farnesol with amphotericin B ($P=0.0124$) and caspofungin ($P=0.0003$), as shown in Table 1. The

antifungal MICs obtained against *C parapsilosis* ATCC 22019 were 0.5 mg/L for itraconazole and caspofungin and 1.0 and 0.03 mg/L for amphotericin B and voriconazole, respectively.

Ergosterol quantification

The results showed that the exposure of the 10 strains of *C. posadasii* to sub-inhibitory concentrations of farnesol altered the amount of ergosterol extracted from each sampled strain. Higher concentrations of farnesol resulted in the extraction of smaller amounts of ergosterol from the fungal cells. Similar results were observed with itraconazole, which is known to inhibit ergosterol biosynthesis. Figure 1 shows the geometric means of the obtained results for the ten evaluated isolates.

Osmotic stress

The MIC values obtained by using RPMI medium supplemented with NaCl were significantly lower than the MICs found, when standard RPMI medium was used, as shown in Figure 2. The geometric mean obtained for the itraconazole MICs were 0.25 and 0.00012 mg/L for RPMI-Standard and RPMI + NaCl, respectively.

Discussion

Several studies have confirmed the inhibitory effect of farnesol on the growth of several bacteria (12, 13, 27). More recently, many studies have described the activity of farnesol against different species of fungi (15-18). In this study, for the first time, the antifungal activity of farnesol was investigated against the primary pathogen *C. posadasii*, the causative agent of coccidioidomycosis.

The *in vitro* MIC values obtained showed a high inhibitory activity of farnesol against all tested strains, with no differences between clinical and environmental strains. These results, ranging from 0.00171 to 0.01369 mg/L (0.0078-0.0616 µM), are quite low compared to those previously described for other fungal species. Derengowski et al. (2009) demonstrated the *in vitro* activity of farnesol against the dimorphic fungus *Paracoccidioides brasiliensis*, which presented an average MIC of 25 µM (16). Moreover, recent studies from our group with the species *Cryptococcus neoformans* and *C. gattii* showed MIC values ranging from 0.29 to 75 µM for both species (17), while the farnesol MICs ranged from 9.37 to 150 µM against different *Candida* species, (18).

In this study, we observed that farnesol significantly decreased the MICs for itraconazole and voriconazole and synergistically interacted with amphotericin B and caspofungin. These results corroborate reports previously described in the literature that suggest the potential use of farnesol as an adjuvant in antifungal therapy and its ability to promote the reversal of antimicrobial resistance (8, 28). Cordeiro et al. (2012) showed that farnesol, when combined with antifungal drugs, reversed the *in vitro* antifungal resistance of *Candida* spp. strains and synergistically interacted with all tested drugs (18).

Regarding the mechanism of action of farnesol in the fungal cells, considering that it shares several precursors with ergosterol in the biosynthetic pathway, some authors have suggested that this compound acts by inhibiting the synthesis of ergosterol, causing alterations in the cell membrane (8). The results of this study confirm the above inferences, since the concentration of ergosterol in the cell decreased, when the strains of *C. posadasii* were exposed to farnesol, even at sub-inhibitory doses. The results also showed that exposure to higher the concentrations of farnesol led to the extraction of

lower amounts of ergosterol from fungal cells. A similar effect was also observed when cells were exposed to itraconazole, which is known to inhibit the synthesis of ergosterol. Thus, it can be suggested that the two agents act similarly.

Furthermore, when the strains were subjected to a medium with high salt concentration, the obtained farnesol MICs considerably decreased, supporting the proposition that the mechanism of action of this compound is associated with degeneration of the fungal membrane, since under osmotic stress, the strains have more fragile cells. Derengowski et al. (2009) reported that farnesol does not appear to act in the cell wall, because the wall remains intact in cells of *P. brasiliensis* after exposure to this compound (16).

Considering the high *in vitro* antifungal activity of farnesol against strains of *C. posadasii* and its low toxicity, as previously demonstrated by Navarathna et al. (2007) (29), its use in combination with other drugs as a possible therapeutic antifungal agent and an adjuvant in the treatment of coccidioidomycosis seems feasible. Thus, this work brings perspectives for the performance of *in vivo* studies to further elucidate the effects of farnesol on the host cells.

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312

313 Figure 1. Quantification of ergosterol from 10 strains of *Coccidioides posadasii* after
314 exposure to different concentrations of farnesol (A) and itraconazole (B). This values
315 represent the geometric means of the results obtained for all tested strains. R^2 represents
316 the coefficient of determination.

317 Figure 2. Minimum inhibitory concentration values of farnesol against strains of
318 *Coccidioides posadasii* in the presence and absence of osmotic stress.

Table 1. MIC and FICI values for the combinations of farnesol with the antifungal agents caspofungin. voriconazole and itraconazole against strains of *C. posadasii.*

MIC values Drugs alone			**MIC values Drugs in combination**			**FICI**	**Synergism No of strains**
FNZ mg/L	**Antifungal** mg/L		**FNZ** mg/L	**Antifungal** mg/L		**range**	**(%)**
0.00171- 0.01369	**ITR**	0.125 – 0.5	0.000107– 0.003424	**ITR**	0.0078 – 0.0312	0.125 - 1	12 (66.6%)
	VRZ	0.125 – 0.25	0.000428– 0.001712	**VRZ**	0.0156 – 0.125	0.125 – 2	15 (83.3%)
	CAS	32 – 16	0.000107– 0.003424	**CAS**	1 - 8	0.125 - 1	17 (94.4%)
	AMB	0.0625 – 0.125	0.000428– 0.003424	**AMB**	0.0078 – 0.0312	0.25 - 1	17 (94.4%)

FNZ: farnesol; AMB: amphotericin B; ITR: itraconazole; VRZ: voriconazole; CAS: caspofungin.