Farnesol increases the activity of echinocandins against *Candida auris* biofilms

Short title: Farnesol with echinocandins against *C. auris* biofilms

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Abstract

Candida auris biofilms exhibit decreased susceptibility to echinocandins, which is associated with poorer clinical outcomes. Farnesol is a quorum-sensing molecule enhancing the activity of antifungals; therefore, we evaluated the *in vitro* effect of farnesol with anidulafungin, caspofungin or micafungin against biofilms using fractional inhibitory concentration indices (FICI), Bliss independence model, LIVE/DEAD-assay and scanning electron microscopy. Based on mathematical models, farnesol caused synergism in eleven out of twelve cases (FICIs range 0.133-0.507; Bliss synergy volume range 70.39-204.6 $\mu M^2\%$). This was confirmed by microscope images of combination-exposed biofilms. Our study showed the prominent effect of farnesol with echinocandins against *C. auris* biofilms.

Keywords: *Candida auris*, biofilm, synergy, farnesol, lock therapy

Candida auris differentially adheres to various polymeric surfaces and forms biofilms, which are associated with increased virulence and poorer clinical outcomes. Moreover, they exhibit decreased susceptibility to the traditionally biofilm-active echinocandins (MIC_{90} ranged from 0.25 to >32), which usually show good activity against planktonic *C. auris* (MIC_{90} ranged from 0.25 to 0.5).[1-2] Farnesol is a sesquiterpene alcohol quorum-sensing molecule, which prevents the biofilm formation in *C. albicans* by inhibition of Ras1-cAMP-PKA cascade supporting the hypha-to-yeast transition; in addition, it has relevant anti-biofilm activity against various *Candida* species.[3-5] Given this well-documented inhibitory effect, we hypothesized that it could enhance the activity of echinocandins against *C. auris* biofilms, opening new approaches towards anti-biofilm treatment. We evaluated the *in vitro* effects of farnesol in combination with an echinocandin (anidulafungin, caspofungin or micafungin) against *C. auris* isolates.

C. auris strains, all of the South Asian/Indian lineage[6] were obtained from National Mycology Reference Laboratory, UK. Methodology is detailed in the supplementary material (Supplement 1). Briefly, susceptibility to anidulafungin, caspofungin, micafungin (all from Molcan, Toronto, Canada) and farnesol (Sigma, Budapest, Hungary) was determined by broth microdilution in RPMI-1640 (Sigma, Budapest, Hungary) in accordance with the document CLSI M27-A3 (ranges 0.008 to 4 mg/l for echinocandins and 1.17 to 300 μM for farnesol). MICs were read after 24-hours using the partial inhibition criterion (at least 50% growth reduction compared to growth control) (Supplement 1).[7] The susceptibility of one-day-old biofilms was determined by XTT-assay.[5,8] The tested concentrations for sessile MIC determination ranged from 1 to 64 mg/l and from 1.17 to 300 μM for echinocandins and farnesol, respectively. MICs of biofilms were defined as the lowest drug concentration resulting in at least 50% metabolic activity reduction compared to growth control (Supplement 1). Drug-drug interactions were assessed by checkerboard assays with the fractional inhibitory

concentration index (FICI) interpreted as follows: ≤0.5, synergistic; 0.5 to 4, indifferent; and >4, antagonistic (Supplement 1).[9] The results by FICI were confirmed by MacSynergy™ II drug response curves using the Bliss independence algorithm. MacSynergy™ II generates a three-dimensional response curve of the synergy-antagonism[10]. Peaks and troughs show synergy or antagonism and are significant if their interaction volumes are greater or lower than ±25 μM²% at a confidence interval of 95%.[10] Susceptibility tests and examination of interactions were performed in triplicate and medians were presented. The efficacy of combinations was confirmed by LIVE/DEAD® BacLight™ viability assay as described earlier (Supplement 1).[11] Preformed biofilms were treated as follows: 4 mg/l of the different echinocandins alone, 75 μM farnesol alone, 4 mg/l of a given echinocandin in combination with 75 μM farnesol. The recommended echinocandin dosing strategies produce mean trough concentrations ranges from 1 to 4 mg/l; therefore, clinical conditions can be properly mimicked by tested 4 mg/l.[12] Regarding tested farnesol concentration, 75 μM farnesol was chosen because farnesol MICs were ≤75 μM in combination with echinocandins in eleven out of twelve cases. Morphological changes induced by the different treatments were examined using scanning electron microscope (SEM). Biofilms were grown on 10 mm diameter circular coverslips and prepared for SEM analyses as published previously (Supplement 1).[3] The antifungal treatment was the same as for the LIVE/DEAD assay.

All planktonic *C. auris* isolates were susceptible to tested echinocandins based on tentative MIC breakpoints recommended by CDC (≥4 mg/l for anidulafungin, ≥2 mg/l for caspofungin, ≥4 mg/L for micafungin).[13] The median planktonic MICs ranged from 0.06 to 0.5 mg/l, from 0.5 to 1 mg/l, from 0.12 to 0.25 mg/l and from 150 to 300 μM for anidulafungin, caspofungin, micafungin and farnesol, respectively. The sessile MIC results and FICIs are shown in Table 1. The median sessile MICs observed for anidulafungin, caspofungin and micafungin combined with farnesol were reduce by 64-128-fold, 64-128-fold and 128-fold, respectively (Table 1).

The median sessile MIC values for farnesol showed 2-8-fold, 4-64-fold and 4-8-fold decreases in combination with anidulafungin, caspofungin and micafungin, respectively (Table 1). Synergy was reported both for caspofungin (FICIs 0.156-0.5) and micafungin (FICIs 0.133-0.281). In case of anidulafungin, synergy was observed for three out of four isolates (FICIs 0.14-0.375), indifferent interaction was found exclusively with isolate 10; however, the median FICI was very close to the synergy threshold (FICI 0.507) (Table 1). The results by FICI were confirmed by the Bliss model, where farnesol produces 70.39 $\mu M^2\%$, 204.6 $\mu M^2\%$ and 139.47 $\mu M^2\%$ synergy volume (synergy threshold >25 $\mu M^2\%$) in combination with anidulafungin, caspofungin and micafungin, respectively (Supplement 2A-C). LIVE/DEAD viability staining (Figure 1A-H) revealed that the biofilms exposed to any of the three echinocandins exhibited increased cell death in the presence of farnesol (Figure 1F-H) compared to untreated sessile cells (Figure 1A) as confirmed by quantitative culturing of biofilms as well (data not shown), while farnesol alone did not influence the live-dead ratio (Figure 1B). SEM images were in line with results of the LIVE/DEAD viability assay (Figure 1I-P). The most prominent inhibition was observed in case of anidulafungin with farnesol as confirmed by Figure 1F and N.

Biofilm-forming ability of *C. auris* is well-documented; however, its role in catheter-associated candidiasis remains obscure.[1] Recently, echinocandins became the first-line therapy for *C. auris* infections because the MIC_{50} values for echinocandins ranged from 0.125 to 1 mg/l for planktonic cells and rate of echinocandin resistance is relatively low (2% to 8%).[14-16] However, a recent report demonstrated that the activity of echinocandins is significantly lower against *C. auris* than against *C. albicans* biofilms[1], leaving clinicians devoid of any provenly efficient therapeutic option against *C. auris* biofilms. Though this warrants testing of efficacy of combinations, studies focusing on combination-based therapy against *C. auris* are scant[17]; in addition, drug-drug combinations with anti-biofilm effect are also understudied. Wall *et* al. (2018) recently examined the adjuvant effect of multiple putative potentiating agents in

combination with traditional antifungals against *C. auris* biofilms, and only ebselen showed synergy with frequently used antifungals *in vitro*.[18] The current study revealed a synergy between the tested echinocandins and farnesol against *C. auris* biofilms. The mechanism of this synergy is not fully understood. Farnesol was found to modulate the expression of genes linked to ergosterol biosynthesis in several *Candida* species.[19] Therefore, detrimental effects on the cell wall and imbalance of ergosterol synthesis may occur simultaneously caused by echinocandins and farnesol, respectively,[3,19] causing simultaneous damage to the cell wall and the cell membrane. Furthermore, farnesol can induce reactive oxygen species production and disruption of mitochondrial function as described in *C. albicans*, which may enhance the activity of echinocandins.[19] It is noteworthy that the systemic usage of several observed effective farnesol concentrations may be contraindicated due to emerging toxicity.[20] However, they may enhance the activity of echinocandins as a potential adjuvant in certain alternative treatments as lock therapy similar to *C. albicans* or *C. parapsilosis*.[4-5] This anti-biofilm strategy avoids systemic toxicity since farnesol only acts within catheter.

In conclusion, farnesol enhanced the activity of echinocandins against the multidrug-resistant *C. auris in vitro* suggesting an alternative approach to overcome the observed echinocandin resistance of *C. auris* biofilms. This calls for *in vivo* studies with farnesol to test its utility as a potentiator of echinocandins in catheter lock therapy model.

Acknowledgements

The authors thank Dr. Gábor Kardos for a critical review of the manuscript.

Conflict of interest

L. Majoros received conference travel grants from MSD, Astellas and Pfizer. All other authors declare no conflicts of interest.

Funding

The study was supported by the EFOP-3.6.3-VEKOP-16-2017-00009 program. Zoltán Tóth and Fruzsina Nagy were supported by the ÚNKP-18-3 New National Excellence Program of the Ministry of Human Capacities.

Ethical approval

Not required

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Table 1 Minimum inhibitory concentration of anidulafungin (ANI), caspofungin (CAS) and micafungin (MICA) alone and in combination with farnesol (FAR) against *Candida auris* biofilms (sMIC). Furthermore, *in vitro* interactions (INT) by fractional inhibitory concentration index (FICI) of anidulafungin, caspofungin and micafungin in combination with farnesol against *Candida auris* biofilms. All tests were performed in triplicate and the median values were presented.

Isolates No.	**Median sMIC values**				**Type of interaction**	
	sMIC alone		**sMIC in combination**		**Median FICI**	
	ANI (mg/l)	**FAR (μM)**	**ANI (mg/l)**	**FAR (μM)**	**ANI**	**INT**
10	>64	300	1	150	0.507	Indifferent
12	>64	300	1	75	0.265	Synergy
27	64	300	1	75	0.375	Synergy
82	64	>300	1	75	0.14	Synergy
	CAS (mg/l)	**FAR (μM)**	**CAS (mg/l)**	**FAR (μM)**	**CAS**	**INT**
10	>64	300	1	75	0.5	Synergy
12	64	300	1	37.5	0.25	Synergy
27	>64	300	1	75	0.5	Synergy
82	>64	>300	1	9.375	0.156	Synergy
	MICA (mg/l)	**FAR (μM)**	**MICA (mg/l)**	**FAR (μM)**	**MICA**	**INT**
10	>64	300	1	75	0.265	Synergy
12	>64	150	1	37.5	0.281	Synergy
27	>64	300	1	75	0.258	Synergy
82	>64	>300	1	75	0.133	Synergy

[a] MIC is offscale at >64 mg/l, 128 mg/l (one dilution higher than the highest tested concentration) was used for analysis

[b] MIC is offscale at >300 μM, 600 μM (one dilution higher than the highest tested concentration) was used for analysis

Figure 1

LIVE/DEAD fluorescence imaging of one representative *Candida auris* isolate (isolate 82). Pictures A and B show the untreated one-day-old biofilm and farnesol-exposed (75 μM) sessile cells respectively. Pictures C, D and E demonstrate the anidulafungin, caspofungin -and micafungin-exposed biofilms (4 mg/L), respectively; while images F, G and H show the anti-biofilm effect of anidulafungin, caspofungin and micafungin (4 mg/L) in the presence of farnesol (75 μM), respectively. Live cells (green) and nonviable cells (red) were stained with Syto9 and propidium iodide, respectively. All images show typical fields of view. Scale bars represent 10 μm.

Pictures from I to P show scanning electron microscopic images of preformed *Candida auris* biofilms (isolate 82). Biofilms were grown on circular coverslip for 24 hours prior to treatment with different agents. Afterwards, the treated discs were incubated for 24 hours in RPMI-1640 containing 75 μM farnesol (J), 4 mg/L anidulafungin (K), 4 mg/L caspofungin (L), 4 mg/L micafungin (M), 4 mg/L anidulafungin with 75 μM farnesol (N), 4 mg/L caspofungin with 75 μM farnesol (O) or 4 mg/L micafungin with 75 μM farnesol (P) and compared to untreated biofilm (I). All images represent typical fields of view. Scale bars represent, 20 μm.