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The *in vitro* and *in vivo* efficacy of fluconazole in combination with farnesol against *Candida albicans* isolates using a murine vulvovaginitis model

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Farnesol is a quorum-sensing molecule that inhibits biofilm formation in *Candida albicans*. Previous *in vitro* data suggest that, in combination with certain antifungals, farnesol may have an adjuvant anti-biofilm agent. However, the *in vivo* efficacy of farnesol is very questionable. Therefore, the *in vitro* and *in vivo* activity of fluconazole combined with farnesol was evaluated against *C. albicans* biofilms using fractional inhibitory concentration index (FICI) determination, time-kill experiments and a murine vulvovaginitis model. The median biofilm MICs of fluconazole-sensitive *C. albicans* isolates ranged between 4 -> 512 mg/L and 150–300 μM for fluconazole and farnesol, respectively. These values were 512 -> 512 mg/L and > 300 μM for fluconazole-resistant clinical isolates. Farnesol decreased the median MICs of fluconazole by 2-64-fold for biofilms. Based on FICI, synergistic interaction was observed only in the case of the sessile SC5314 reference strain (FICIs: 0.16–0.27). In time-kill studies, only the 512 mg/L fluconazole and 512 mg/L fluconazole + 75 μM farnesol reduced biofilm mass significantly at each time point in the case of all isolates. The combination reduced the metabolic activity of biofilms for all isolates in a concentration- and time-dependent manner. Our findings revealed that farnesol alone was not protective in a murine vulvovaginitis model. Farnesol was not beneficial in combination with fluconazole for fluconazole-susceptible isolates, but partially increased fluconazole activity against one fluconazole-resistant isolate, but not the other one.

***Keywords*:** time-kill experiment, FIC index, murine vulvovaginitis, fluconazole-resistant, synergy

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Introduction

Farnesol is an endogenous quorum-sensing molecule influencing fungal morphogenesis and biofilm production (Hornby *et al.*, 2001). Farnesol blocks yeast-hypha transition and thus decreases biofilm formation in the case of *Candida albicans* in a concentration-dependent manner (Ramage *et al.*, 2002; Langford *et al.*, 2009).

In vitro synergy between several antifungal agents and farnesol against biofilms has been reported in the case of the *C. albicans* SC5314 reference strain (Katragkou *et al.*, 2015). These data suggest that farnesol in combination with antifungals including fluconazole may have utility as an adjuvant anti-biofilm agent. However, little information is available regarding clinical isolates and the interaction between farnesol and antifungals against *C. albicans in vivo* (De Cremer *et al.*, 2015). As previously described, *C. albicans* forms biofilm *in vivo* on the surface of the vaginal epithelium (Harriott *et al.*, 2010). The integrity of *in vivo* biofilms may be disrupted by the co-administration of fluconazole and farnesol; the latter induces degradation of the biofilm at supraphysiological concentrations (300 μM), which may provoke cell detachment or dispersion and the detached cells may be eliminated more effectively by fluconazole (Uppuluri *et al.*, 2010).

Therefore, the aim of this study was to determine the *in vitro* activity of fluconazole with and without farnesol against biofilms formed by fluconazole-sensitive and fluconazole-resistant clinical isolates of *C. albicans*. Furthermore, we evaluated the *in vivo* effect of the combination treatment on the vaginal fungal burden in a murine vulvovaginitis model.

Materials and Methods

Organisms

Two fluconazole-sensitive (1216, 10431) and two fluconazole-resistant (21616, 27700) isolates isolated from vulvovaginal candidiasis were used in this study together with the reference strain SC5314. Clinical isolates were identified by API ID32C panel as well as by matrix-assisted laser desorption/ionization time of flight mass spectrometry (Microflex, Bruker Daltronics).

Susceptibility testing of planktonic cells

Antifungal susceptibility of *Candida* isolates to fluconazole (Sigma) and to farnesol (Sigma) was tested using the broth microdilution method in RPMI-1640 (with L-glutamine and

without bicarbonate, pH 7.0 with MOPS; Sigma) in accordance with the CLSI standard M27-A3 protocol (Clinical and Laboratory Standards Institute, 2008). The final concentrations of the drug ranged between 0.06–4 mg/L and 2–128 mg/L for fluconazole in the case of susceptible and resistant isolates, respectively, and between 1.17–300 μM for farnesol in the case of all isolates.

Farnesol was obtained as 3M stock solution which was diluted to a 30 mM working stock solution in 100% methanol. A single lot of farnesol was used throughout the study. The working concentrations were prepared in RPMI-1640. Each drug-free control well contained 1% (vol/vol) methanol (Katragkou *et al.*, 2015).

Susceptibility testing for planktonic cells was carried out in 96-well plates at 37°C for 24 h. The inoculum was 0.5–2.5 × 10^3 cells/ml. Minimum inhibitory concentrations (MICs) were defined based on turbidity (492 nm) being at least 50% growth reduction compared with drug-free control. Percentage change in turbidity was calculated on the basis of absorbance (A) as $100\% \times (A_{well} - A_{background})/(A_{drug\text{-}free\ well} - A_{background})$. The background was measured from the fungus-free well. All isolates were tested in three independent experiments and the median of the three values was used in the analysis (Zhou *et al.*, 2012; Katragkou *et al.*, 2015).

Biofilm mass determination

Biofilms were prepared as described by Pierce *et al.* (2008). One-day-old biofilm mass was quantified as previously described by O'Toole (2011). In brief, 125 μl of a 0.1% crystal violet solution was added to each well containing prewashed biofilms and incubated for 15 min at room temperature. The solution was then removed and plates were washed three times with 200 μl physiological saline to remove excess crystal violet completely. Afterwards 125 μl of a 30% acetic acid solution was added to each well to solubilize the biofilm-bound crystal violet. After 15 min incubation at room temperature, 100 μl supernatant was transferred to a new 96-well plate and read spectrophotometrically at 540 nm. Blank wells contained 100 μl of 30% acetic acid (O'Toole, 2011).

Susceptibility testing of biofilms

The activity of fluconazole and farnesol against biofilms was evaluated using the XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) reduction assay (Hawser, 1996; Zhou *et al.*, 2012; Katragkou *et al.*, 2015). Based on our preliminary results the concentrations tested in MIC determination in biofilms were 8–512 mg/L and 1.17–300 μM for fluconazole and farnesol, respectively. To determine the 24-h biofilm MICs, the one-day-old biofilms were first washed three times with 200 μl sterile physiological saline. All wells, including the blank ones, were filled with 100 μl of 0.5 g/L XTT/1 μM menadione solution. The plates were covered with aluminium foil and incubated at 37°C for 2 h. After incubation, 80 μl of the supernatant was removed and transferred into a new 96-well plate in order to measure the absorbance spectrophotometrically at 492 nm. MICs were defined as the lowest concentration that produced at least 50% reduction in metabolic activity of fungal biofilms. The percentage change in metabolic activity was calculated in the same way as described for the measurement of turbidity for planktonic cells. All isolates were tested in three independent experiments and the median of the three values was used in the analysis (Hawser, 1996; Zhou *et al.*, 2012; Katragkou *et al.*, 2015).

Interactions between farnesol and fluconazole

A fractional inhibitory concentration index (FICI) as a non-parametric approach was used to evaluate drug-drug interactions using a two-dimensional broth microdilution checkerboard assay both for planktonic and sessile cells (Meletiadis *et al.*, 2005; Zhou *et al.*, 2012; Katragkou *et al.*, 2015). The concentration ranges were same as described above for MIC determination against planktonic cells and biofilms. The FICI model expressed as $\Sigma FIC = FICA + FICB = MIC_A^{comb}/MIC_A^{alone} + MIC_B^{comb}/MIC_B^{alone}$, where MIC_A^{alone}, and MIC_B^{alone} are the MIC values of agents A and B used alone and MIC_A^{comb} and MIC_B^{comb} are the MICs of agents A and B at the isoeffective combinations, respectively. FICI was defined as the lowest ΣFIC. The MIC values of the drugs alone and of all isoeffective combinations were determined as the lowest drug concentrations showing at least 50% reduction of turbidity for planktonic, or at least 50% reduction of metabolic activity for sessile cells compared to the untreated controls. Off-scale MICs were converted to the next highest two-fold concentration. The interaction between fluconazole and farnesol was interpreted as synergistic when FICI was ≤ 0.5, as indifferent interaction when FICI was between > 0.5 and 4 and as antagonism when FICI was > 4 (Meletiadis *et al.*, 2005; Zhou *et al.*, 2012; Katragkou *et al.*, 2015).

Time-kill experiments

Time-kill experiments were carried out with biofilms to examine the effect of fluconazole alone and in combination with farnesol on biofilm mass and metabolic activity on separate plates (Zhou *et al.*, 2012; Kovács *et al.*, 2016). After the biofilms were prepared, predetermined wells were assigned to endpoints of 3, 6, 9, 12, and 24 h, then the different drug concentrations were added in RPMI-1640 to all wells at time 0. After 3, 6, 9, 12, and 24 h, the corresponding pre-assigned wells were washed in both plates and the biofilm mass as well as the metabolic activity of biofilms was measured as described above. Baseline biofilm mass and metabolic activity was measured from wells assigned as such prior to adding the drug (Zhou *et al.*, 2012; Kovács *et al.*, 2016). All isolates were tested in three independent experiments.

The drug concentrations tested in the time-kill experiments were 0.5 mg/L, 8 mg/L, 64 mg/L, and 512 mg/L fluconazole with and without 75 μM farnesol against the fluconazole-sensitive strains and against the reference strain as well as 64 mg/L, 128 mg/L, 256 mg/L, and 512 mg/L fluconazole with and without 75 μM farnesol against the two resistant strains. The farnesol concentration of 75 μM was chosen as this was the lowest farnesol concentration which consistently displayed a measurable effect in the checkerboard microdilution.

Time-kill curves were prepared from the measured biofilm mass and metabolic activity values using GraphPad Prism

Table 1. Minimum inhibitory concentrations (MICs) of fluconazole alone and in combination with farnesol against fluconazole sensitive (S) and resistant (R) *Candida albicans* planktonic cells

Isolate	Median MIC (range) of drug used				FICI[b]	Interaction
	Alone		In combination			
	Fluconazole (mg/L)	Farnesol (μM)	Fluconazole (mg/L)	Farnesol (μM)		
SC5314 (S)	0.5	> 300[a]	1 (0.5–1)	2.34 (1.17–300)	2.00 (1.00–2.5)	Indifferent
1216 (S)	0.5	> 300[a]	1 (0.5–1)	2.34 (1.17–2.34)	2.00 (1.00–2.00)	Indifferent
10431 (S)	0.5	> 300[a]	1	1.17 (1.17–2.34)	2.00	Indifferent
27700 (R)	16	> 300[a]	32 (16–32)	1.17	2.00 (1.00–2.00)	Indifferent
21616 (R)	32	> 300[a]	64	1.17	2.00	Indifferent

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

6.05. One-way ANOVA with Dunnett's post-testing was used to analyse the biofilm mass and the reduction in metabolic activity caused by the drug alone and in combinations compared with the drug-free control. Concentrations with and without farnesol were compared with each other using one-way ANOVA with Sidak's post-testing. Significance was defined as $P < 0.05$.

In vivo model

BALB/c immunocompetent female mice (22–23 g) (Charles River) were used. The animals were maintained in accordance with the Guidelines for the Care and Use of Laboratory Animals. The experiments were approved by the Animal Care Committee of the University of Debrecen, Debrecen, Hungary (permission no. 12/2014 DEMÁB).

Mice were administered 50 μl subcutaneous estradiol-valerat (10 mg/ml in sesame seed oil) three days prior to vaginal infection (Fidel *et al.*, 2000; Hisajima *et al.*, 2008; Kovács *et al.*, 2014). In accordance with our preliminary experiments, animals were infected intravaginally with 1–1.2 × 10^7 CFU in 25 μl physiological saline. Inoculum density was confirmed by plating serial dilutions on Sabouraud dextrose agar. Mice were divided into seven groups (10 mice per group); i) control; ii) 5 mg/kg/day; iii) 35 mg/kg fluconazole in a single dose; iv) 150 μM/day farnesol; v) 300 μM/day farnesol; 5 mg/kg/day fluconazole combined vi) with 150 μM/day farnesol and vii) with 300 μM/day farnesol. All regimens were started 24 h postinfection. Fluconazole doses were administered intraperitonally at a volume of 0.5 ml, while farnesol was given intravaginally at a volume of 25 μl one h after the fluconazole treatment. Farnesol was diluted in 0.25% Tween 80 solution. Control mice were given 0.5 ml physiological saline intraperitonally as well as 25 μl 0.25% Tween 80 solution without farnesol intravaginally (Hisajima *et al.*, 2008). At 4 days postinfection, mice were euthanized and their vaginae were excised, weighed and homogenized aseptically. Fungal tissue burden was determined by quantitative culturing. Vaginal burden was analyzed using Kruskal-Wallis test with Dunn's post-test (GraphPad Prism 6.05.). Significance was defined as $P < 0.05$.

Results

MIC results for planktonic cells

The planktonic MIC values of fluconazole alone and combined with farnesol are summarized in Table 1. Two out of four tested planktonic clinical isolates and the reference strain were susceptible to fluconazole according to CLSI breakpoints. Isolates 27700 and 21616 were resistant (Pfaller *et al.*, 2010). Farnesol had no effect alone regardless of the fluconazole susceptibility of planktonic isolates. The MICs of farnesol in combinations decreased, however it was primarily the effect of fluconazole that dominated in these combinations (Table 1).

MIC results for biofilms

All tested isolates formed biofilm based on the crystal violet assay. The median values and ranges of MICs for sessile *C. albicans* cells are shown in Table 2. The MIC values of drugs varied more widely compared to planktonic cells. Farnesol caused a 2- to 64-fold decrease in the median fluconazole MICs against biofilms. Furthermore, median farnesol MIC

Table 2. Minimum inhibitory concentrations (MICs) of fluconazole alone and in combination with farnesol against fluconazole sensitive (S) and resistant (R) *Candida albicans* biofilms

Isolate	Median MIC (range) of drug used				FICI[c]	Interaction
	Alone		In combination			
	Fluconazole (mg/L)	Farnesol (μM)	Fluconazole (mg/L)	Farnesol (μM)		
SC5314 (S)	512	300	8	75 (37.5–75)	0.27 (0.16–0.27)	Synergism
1216 (S)	4 (2–8)	150 (150–300)	0.5 (0.12–4)	75 (37.5–150)	0.63 (0.38–1)	Indifferent
10431 (S)	> 512[a]	150	512	37.5	0.75	Indifferent
27700 (R)	512	> 300[b]	256	300	1	Indifferent
21616 (R)	> 512[a]	> 300[b]	256	300	0.75	Indifferent

[a] MIC is offscale at > 512 mg/L, 1024 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
[c] Fractional Inhibitory Concentration Index

values exhibited 2- to 4-fold reduction in combination with fluconazole for sessile cells (Table 2).

Interactions between farnesol and fluconazole by FICI

The results of fluconazole-farnesol interaction based on FICI are summarized in Table 1 and Table 2 for planktonic and sessile cells, respectively. Only indifferent interactions were detected in the case of planktonic isolates (Table 1). For biofilms, synergy between fluconazole and farnesol was observed only against the reference strain SC5314. Against other sessile clinical isolates, the interaction was indifferent, similarly to the findings with planktonic forms (Table 2). Antagonism was never observed (FICIs < 4) (Tables 1 and 2).

Time-kill results with biofilm mass

Interactions as tested by time-kill investigations are shown in Fig. 1. Only the highest tested fluconazole concentration without farnesol (512 mg/L) decreased the biofilm mass of fluconazole sensitive cells significantly between 3 and 24 h, as compared to the control biofilms ($P < 0.05$–0.01) (Fig. 1A). Among the combinations, the 512 mg/L fluconazole combined with 75 μM farnesol showed a significant reduction in biofilm formation against all sensitive *C. albicans* isolates at each tested time point ($P < 0.05$–0.001). There were no significant differences between the given concentrations alone and the combinations (0.5 mg/L vs. 0.5 mg/L + 75 μM, etc.) ($P > 0.05$) (Fig. 1A). Against resistant isolates, only 512 mg/L alone as well as in combination with 75 μM farnesol decreased the biofilm mass significantly between 3 and 24 h as compared to the control curve ($P < 0.05$–0.001). At 24 h, fluconazole alone and the corresponding combination with farnesol always showed statistically comparable efficacy for all tested concentrations ($P > 0.05$) (Fig. 1B).

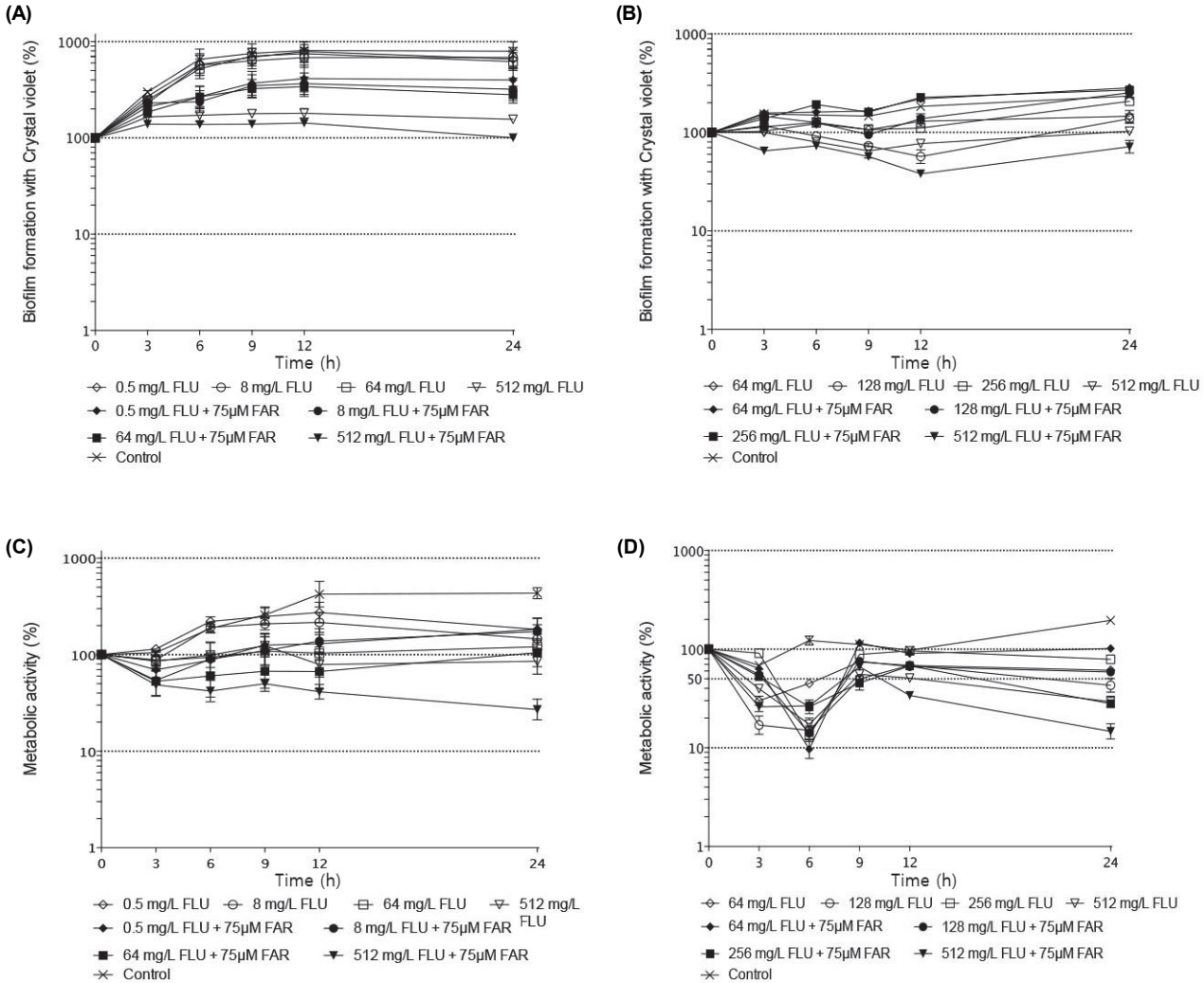

Fig. 1. *In vitro* efficacy of fluconazole with and without farnesol in time-kill experiments. The time-kill curves show the effect exerted by fluconazole alone and in combination with farnesol against three fluconazole-sensitive *Candida albicans* isolates (SC5314, 1216, 10431) (A, C) and against two resistant isolates (21616, 27700) (B, D). Plots A and B show changes in biofilm mass, plots C and D show changes in metabolic activity. All three fluconazole susceptible isolates behaved similarly, therefore the percentage averages of the three individual isolates are shown in the same time-kill plots, where each time point represents the mean ± SEM (standard error of mean). The two resistant isolates also showed similar results, and the plots are prepared as for the susceptible isolates.

Time-kill results for metabolic activity

Concentration- and time-dependent action of the drugs on the metabolic activity of biofilms was observed for all three fluconazole-sensitive *C. albicans* isolates (Fig. 1C). Significant reduction was observed for all tested fluconazole concentrations (alone and in combination) at 24 h compared with the control curve ($P < 0.01$–0.001), while 512 mg/L fluconazole + 75 μM farnesol was effective even at 6 h ($P < 0.05$) (Fig. 1C). There were no significant differences between the given concentrations alone and the corresponding combinations (0.5 mg/L vs. 0.5 mg/L+75 μM, etc.) ($P > 0.05$) (Fig. 1C).

In the case of resistant isolates, a marked reduction in metabolic activity was observed at 6 h for all tested fluconazole concentrations ($P < 0.001$) (Fig. 1D). After this initial drop, metabolic activity showed transitional increase between 6 and 12 h, but at 24 h significantly reduced metabolic activity was observed for treated cells compared to the control biofilm for all concentrations ($P < 0.001$). At 24 h, only the 256 mg/L + 75 μM caused significantly higher reduction in metabolic activity compared to the corresponding concentration alone (256 mg/L) ($P > 0.001$) (Fig. 1D).

In vivo experiments

Results of the *in vivo* experiments are shown in Fig. 2. Against both fluconazole-susceptible *C. albicans* clinical isolates (1216, 10431), 5 mg/kg/day and the single dose of 35 mg/kg proved to be able to decrease the vaginal fungal burden significantly ($P < 0.05$–0.001). Furthermore, the regimens 5 mg/kg/day with 150 μM/day and 300 μM/day farnesol were also effec-

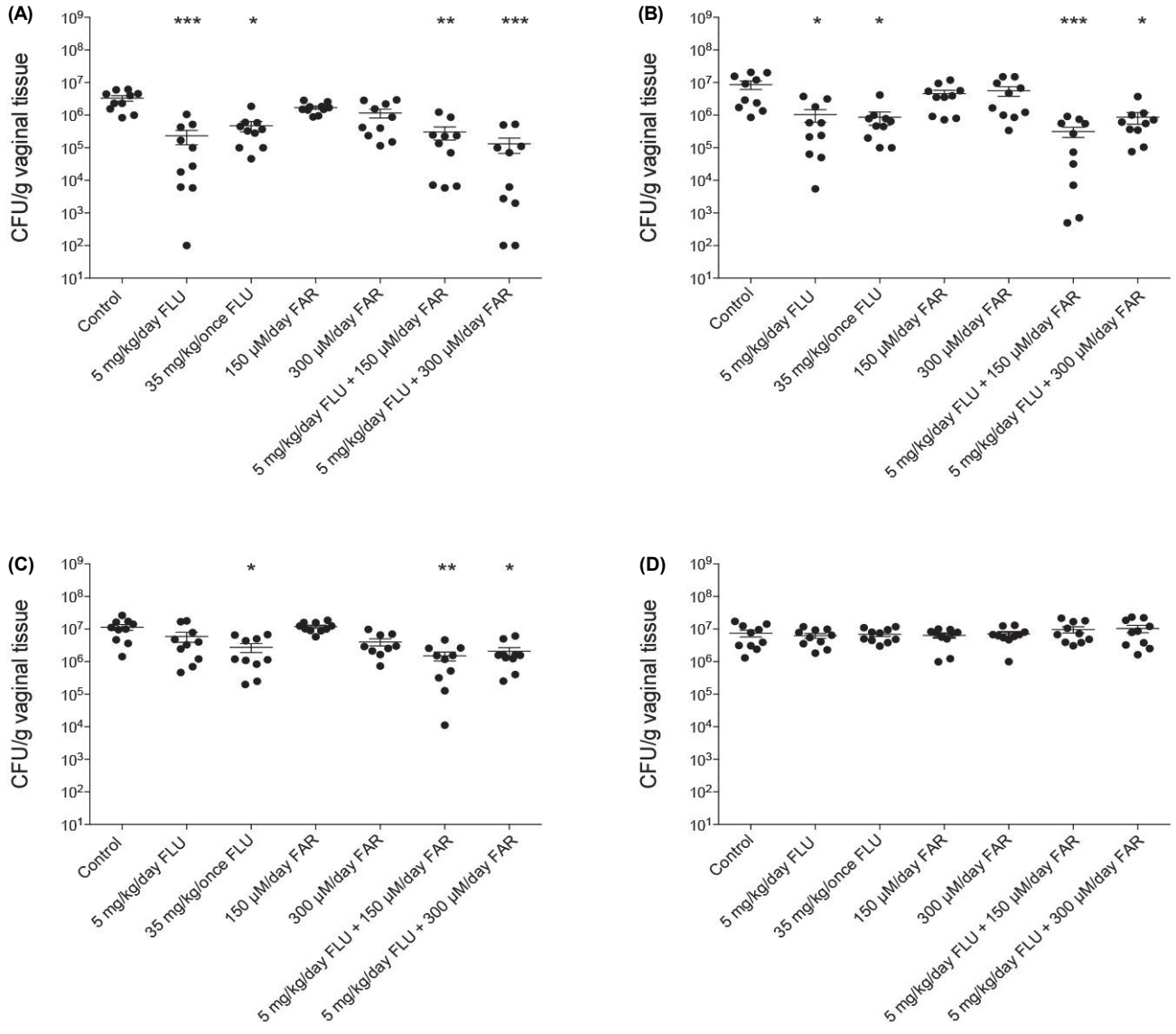

Fig. 2. *In vivo* efficacy of fluconazole with and without farnesol using mouse vulvovaginitis model. Vaginal tissue burden of BALB/c mice infected intravaginally by fluconazole-susceptible *Candida albicans* 1216 (A), *Candida albicans* 10431 (B), and fluconazole-resistant *Candida albicans* 21616 (C), and *Candida albicans* 27700 (D) isolates. The bars represent the mean ± SEM (standard error of mean). Level of statistical significance is indicated at $P < 0.05$ (*), $P < 0.01$ (**), and $P < 0.001$ (***).

tive as compared to control mice ($P < 0.05–0.001$). Neither farnesol doses were effective alone compared to untreated mice ($P > 0.05$) (Fig. 2A and B). Effective treatment arms were statistically comparable ($P > 0.05$).

Against the fluconazole-resistant isolate 21616, the regimen of 5 mg/kg/day fluconazole was ineffective, but when combined with farnesol it reduced vaginal burdens significantly, regardless of farnesol dose ($P < 0.05–0.01$). The higher fluconazole dose (35 mg/kg mouse dose, approximately corresponding to the normal human dose of 150 mg based on 24h-AUC) was effective alone (Louie *et al.*, 1999). In summary, farnesol enhanced the *in vivo* efficacy of 5 mg/kg/day fluconazole. Nevertheless, in multiple comparisons none of the combinations were superior to the single dose of 35 mg/kg fluconazole ($P > 0.05$) (Fig. 2C).

Against the fluconazole-resistant 27700 *C. albicans* isolate, none of the regimens were effective ($P > 0.05$) (Fig. 2D). The reference strain SC5314 produced vaginal burdens in control mice 3-log lower than the infective dose, suggesting that the strain had low vaginal virulence, which precluded comparability to the clinical isolates.

Discussion

The medical importance of biofilms formed on biotic surfaces has become increasingly evident (Harriott *et al.*, 2010; Ganguly *et al.*, 2011). Poor activity of azoles against fungal biofilms is a well-known phenomenon, which may decrease efficacy of azole treatment (Pierce *et al.*, 2013). Previous studies described that farnesol shows *in vitro* synergistic interaction with different azoles against *C. albicans*. Cordeiro *et al.* (2013) observed synergism between fluconazole and farnesol in the case of planktonic fluconazole-resistant *C. albicans* isolates (FICI: 0.015–0.374). Sharma and Prasad (2011) observed that farnesol showed *in vitro* synergism in combination with fluconazole, itraconazole, and miconazole against azole-sensitive as well as against azole-resistant planktonic *C. albicans* isolates, including the reference strain SC5314. Katragkou *et al.* (2015) reported synergistic interaction between fluconazole and farnesol using FICI and the Bliss independence model against the biofilm of *C. albicans* SC5314. It is noteworthy, however, that the mean of ΣFICs measured against *C. albicans* SC5314 was very close to the indifference threshold (mean ΣFIC: 0.5 ± 0.02) (Katragkou *et al.*, 2015). In contrast to data of Cordeiro *et al.* (2013) and Sharma and Prasad (2011), the present study demonstrates indifferent interaction for all tested planktonic isolates, including the same reference strain (SC5314) used by Katragkou *et al.* (2015) in their biofilm-based study. As inhibition of *C. albicans* by fluconazole was shown to induce elevated (up to tenfold) endogenous farnesol production (Hornby *et al.*, 2004) that may explain the variations in the interactions between fluconazole and farnesol *in vitro* which were reported by the authors cited above (Sharma and Prasad, 2011; Cordeiro *et al.*, 2013). However, the present study, similarly to the findings of Katragkou *et al.* (2015), reports *in vitro* synergistic interaction against sessile SC5314 as shown by FICI. Notably, this was never observed against biofilms formed by clinical isolates, where only indifferent interactions were detected.

In the case of sessile azole-resistant isolate 27700, checkerboard microdilution revealed paradoxically enhanced metabolic activity with high fluconazole and farnesol concentrations as compared to control wells. It was reported previously that exogenous farnesol exposure may up-regulate genes of the central carbon-metabolic pathways including glycolysis, gluconeogenesis, acetyl-CoA pathway, nitrogen metabolism and amino acid biosynthesis, which may explain the observed paradoxical enhancement of metabolic activity (Han *et al.*, 2012). Presumably, this phenomenon occurred in time-kill experiments with the resistant isolates (21616, 27700) accounting for the transitionally increased metabolic activity observed between 6–12 h.

The *in vivo* effect of farnesol has received much attention recently, but the issue is strongly controversial. The earliest study by Navarathna *et al.* (2007) concluded that farnesol production may act as a virulence factor, as exogenous farnesol (20 mM/mouse) enhanced the mortality in intravenously challenged mice for both intraperitoneal and oral farnesol administration. These data suggest that farnesol may play a key role in fungal pathogenesis. In contrast, Hisajima *et al.* (2008) described a protective effect of farnesol (9 μM/mouse) against oral mucosal candidiasis in a mouse model. They noticed systemic effects in farnesol-treated mice as well (i.e. moderate bodyweight reduction and smaller number of *C. albicans* cells in faeces). Notably, there was a thousand-fold difference between the doses exerting enhancer (20 mM) and protective (9 μM) effects. A similar protective effect of a cocktail of *Candida* regulatory alcohols (containing nanomolar amounts of farnesol) was reported by Martins *et al.* (2012) in a murine model of disseminated candidiasis.

In our experiments, the mean vaginal burden of control mice on day four postinoculation is consistent with values from previous studies (González *et al.*, 2007; Elizondo-Zertuche *et al.*, 2015). Based on our *in vivo* findings, neither 150 μM nor 300 μM farnesol alone had a protective effect against vaginal *Candida* infection, in contrast to the findings of Hisajima *et al.* (2008).

However, against the fluconazole-resistant isolate 21616, both farnesol regimens administered enhanced the activity of 5 mg/kg daily fluconazole significantly as compared to the untreated control mice. Previously, similar reversal of resistance was observed *in vitro* in planktonic cells (Jabra-Rizk *et al.*, 2006; Cordeiro *et al.*, 2013). Nevertheless, the clinical importance of farnesol as a potential adjuvant remains questionable (21616 was the only isolate in this study where farnesol was able to enhance the *in vivo* activity of fluconazole and the effect was relatively small) and seems to be confined to fluconazole-resistant isolates. Furthermore, this resistance reversal may be restricted to some but not all azole-resistant strains.

The only fluconazole-susceptible wild-type isolate against which farnesol enhances the *in vitro* activity of fluconazole consistently is the reference strain SC5314. This *in vitro* synergy reported by Sharma and Prasad (2011) in the planktonic phase and Katragkou *et al.* (2015) in the case of sessile cells as well as by the present study seems to be an exceptional characteristic of the reference strain SC5314 rather than a rule applicable to many or all clinical isolates.

In summary, this is the first study to our knowledge that ex-

amines the effect of farnesol alone and in combination with fluconazole *in vivo*. This work documented that vaginal administration of farnesol alone did not have protective effect in murine vulvovaginitis and it did not consistently enhance the effect of fluconazole either *in vitro* or *in vivo* against *C. albicans* clinical isolates. This indicates that *in vitro* findings are not directly applicable for predicting *in vivo* utility of farnesol as adjuvant; further *in vivo* experiments are needed to clarify the effect of farnesol in different types of *Candida* infection or against different species.

Conflicts of Interest

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

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