Pravastatin inhibits farnesol production in *Candida albicans* and improves survival in a mouse model of systemic candidiasis

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Candidemia remains a major cause of morbidity and mortality, especially in immunocompromised patients. A strategy of reducing virulence and virulence factors of *Candida* spp. is an attractive approach for the treatment of serious infections caused by these yeasts. Recently, farnesol has been reported to be a quorum-sensing autoinducer, as well as a virulence factor of *C. albicans*. In the present study, we examined the effects of pravastatin, a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase inhibitor on the *in vitro* production of farnesol. In addition, the synergistic effects of pravastatin with fluconazole (FLC) were examined in a mouse model of systemic infections. *In vitro* experiments demonstrated that pravastatin had synergistic activity with FLC as judged by fractional inhibitory concentration index (FICI) and suppression of farnesol production at sub-minimum inhibitory concentrations. Furthermore, significant improvement of survival in systemic infection models was shown with pravastatin supplementation. The survival benefits of pravastatin were correlated with reductions of fungal burden. These data suggest the potential of pravastatin as a supportive therapy against *C. albicans* infections. Synergistic antifungal activity and suppression of HMG-CoA reductase-associated *Candida* virulence factors, including farnesol, may explain, at least in part, the *in vivo* efficacy of pravastatin.

**Keywords** *Candida albicans*, statin, farnesol, systemic candidiasis, mouse

Introduction

The incidence of candidemia has increased over the past several decades due to a growing number of patients at higher risk of infection [1]. In recent years, newer antifungal agents, such as triazoles and echinocandins, have been licensed for the treatment and prevention of these fungal infections [2–4]. In spite of development of these agents, candidemia remains a major cause of morbidity and mortality in health care settings [5]. There is still a need for the discovery and development of new agents and strategies for their use in antifungal therapy [6–8].

Statins are widely used for lowering cholesterol levels through their action on 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase. Yeasts use HMG-CoA reductase for the same enzymatic step as in humans, but in yeasts the main end-product of the pathway is ergosterol rather than cholesterol. Based on these data, several investigators have examined the antifungal activity of statins against clinically important fungi, including *Candida, Cryptococcus*, and *Aspergillus* spp. [9–14]. A possible survival benefit of statin therapy in candidemia had been also reported [15]. Recently, farnesol was identified as a quorum-sensing autoinducer, as well as a virulence factor of *C. albicans* [16]. Notably, farnesol is an isoprenoid intermediate of the
mevalonate pathway, and is produced by dephosphorylation of farnesyl pyrophosphate (Fig. 1). Given that the conversion of HMG-CoA to mevalonate is the rate-limiting step of the mevalonate pathway [17], we hypothesized that blockade of this pathway by statins may suppress farnesol production, resulting in the reduction of virulence of Candida and survival benefits in infection models.

In the present study, we examined the in vitro effects of sub-minimum inhibitory concentrations (sub-MICs) of pravastatin on MICs of flucanazole and farnesol production in C. albicans. In addition, we examined the efficacy of pravastatin in mouse survival of systemic C. albicans infection and fungal burdens in liver and kidneys.

Materials and methods

Strains and antifungal drugs used

Seven clinical isolates of C. albicans were obtained from patients with candidemia at Toho University hospital and identified by CHROM agar Candida (Kanto Chemical, Tokyo, Japan) and VITEK 2 automated identification system (SYSMEX bioMérieux, Tokyo, Japan) using VITEK 2 YST cards. Strain SC5314 (obtained from National Institute of Infectious Diseases, Japan) and ATCC 90028 (obtained from the American Type Culture Collection) were used as reference. All reference and clinical isolates were stored as frozen stocks in 15% glycerol at −80°C and were sub-cultured on Sabouraud dextrose agar (SDA; Eiken Chemical, Tokyo, Japan) at 35°C prior to use. Pravastatin was kindly provided by Daiichi Sankyo (Tokyo, Japan), while flucanazole (FLC) was kindly supplied by Pfizer (Tokyo, Japan). Pravastatin was selected over the synthetic statins such as atorvastatin on the basis that it is a fungal derived inhibitor.

In vitro antifungal susceptibility testing

The broth microdilution method was used for testing C. albicans, and was performed in accordance with Clinical and Laboratory Standards Institute (CLSI) guideline M27-A3 [18], with a final inoculum concentration of 0.5 × 10^3 to 2.5 × 10^3 cells/ml. YM broth (Difco Laboratories, Detroit, MI, USA) and RPMI 1640 (with glutamine and phenol red, without bicarbonate) medium (Gibco BRL, Grand Island, NY, USA) buffered to pH 7.0 with 0.165 M MOPS buffer (Dojindo, Kumamoto, Japan) were used in the studies. YM broth per liter of distilled water contained 3 g of yeast extract, 3 g of malt extract, 5 g of peptone, and 10 g of dextrose. The concentration ranges of pravastatin and FLC in 2-fold dilutions were 1–1,024 μg/ml and 0.125–64 μg/ml, respectively. After 24 h incubation at 35°C, the MIC was determined visually as the lowest concentration of drug showing a prominent reduction of growth (≥50%) compared to the drug-free growth control [18]. C. parapsilosis ATCC 22019 was used as a quality control strain.

Growth inhibition study

Clinical isolate C. albicans 06901B was suspended in YM broth, with or without pravastatin, at a cell density of approximately 1.0 × 10^3 cells per ml. After incubation at 35°C on a shaker at 160 rpm, samples were taken at 1, 3, 7, 11, and 24 h, and viable fungal numbers were determined by spreading an aliquot at each time interval on SDA plates after serial 10-fold dilution.

In vitro synergy testing

In vitro synergy of pravastatin with FLC was determined via the checkerboard technique using C. albicans strains SC5314, ATCC 90028, and 06901B. YM broth and RPMI 1640 medium buffered with MOPS were used as broth media, as described above. Pravastatin was tested at serial 4-fold dilutions at concentrations ranging from 0.25–1,024 μg/ml, whereas FLC was tested at serial 2-fold dilutions at concentrations ranging from 0.008–8 μg/ml. The fractional inhibitory concentration index (FICI) was interpreted as follows: ≤0.5, synergy; >0.5 to ≤4, no interaction; and >4, antagonism [19].

Bioassay of culture supernatant

Candida albicans 06901B was suspended in YM broth with or without pravastatin (0, 4, 16, 32, and 64 μg/ml) at
a cell density of 10^7 cells per ml. The control solution was YM broth with or without pravastatin, without suspended C. albicans. After 24 h of incubation at 35°C on a shaker at 160 rpm, the cultures were centrifuged at 4,750 g for 10 min at room temperature. The supernatant and control solution were decanted and filter-sterilized using cellulose acetate filters (0.22 μm pore size; Corning, Tokyo, Japan). Ten microliters of RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) was added (as a filamentation-inducing reagent) to 90 μl of the supernatant or control solution, and then the morphology of C. albicans 06901B was analyzed.

Detection of farnesol in culture supernatants

The detection of farnesol in culture supernatants was performed as described previously [20]. Briefly, C. albicans 06901B was suspended in 5 l of YM broth with or without pravastatin (64 μg/ml) at a cell density of 10^7 cells per ml and incubated at 35°C with aeration on a shaker at 125 rpm for 24 h. The culture was centrifuged at 15,300 g for 20 min at room temperature. The supernatant was decanted and filter sterilized, then extracted with a one-fifth volume of ethyl acetate. The ethyl acetate was removed under reduced pressure on a rotary evaporator. The residue was examined by thin-layer chromatography (TLC). TLC was performed on aluminum-backed Silica-gel 60 F254 plates (Merck, Tokyo, Japan) using 1:4 ethyl acetate:hexane as the mobile phase. Spots were detected under UV light at 254 nm and quantified using ImageJ software. The standard was purchased from Sigma (Tokyo, Japan) as (E, E)-farnesol (96%).

Animals

C3H/HeN mice (female, 7 weeks old) were purchased from Charles River Co. (Atsugi, Japan). Mice were quarantined for one week after reception. Animals were housed individually at constant temperature (23°C) with a 12:12 h light/dark cycle and provided with standard laboratory food and water ad libitum. All animal experiments were performed under the approval of the animal center of Toho University (approval number 11-53-54).

Effects of systemic administration of pravastatin

A total of 38 mice were injected with C. albicans 06901B (5 x 10^4 CFU/mouse), administered intravenously (IV) through the lateral tail vein (day 0). Sixteen mice (n = 8 per group) were used for survival monitoring. Pravastatin was administered intraperitoneally (IP) at 50 mg/kg/day from day 0 for 5 days. Sterile saline was used as a control. Twenty-two mice (n = 11 per group) were used for evaluation of fungal burdens in liver and kidneys. Surviving mice (control group: n = 5; pravastatin group: n = 9) were sacrificed at day 12 by CO₂ asphyxia. The liver and kidneys were removed and homogenized in 1 ml of saline. Homogenates were subjected to serial 1:10 dilutions and were inoculated (10 μl/plate) on SDA plates to determine C. albicans burdens.

Effects of combination treatment with pravastatin and FLC

After IV inoculation of C. albicans 06901B (5 x 10^5 CFU/mouse), mice (n = 16 per group) were administered FLC (4 mg/kg/day) with or without pravastatin (50 mg/kg/day) IP once a day for 5 days (days 5–9). Twenty-two mice (n = 11 per group) were used for monitoring of survival. Ten mice (n = 5 per group) were used for evaluation of fungal burdens in liver and kidneys. Surviving mice (FLC alone group: n = 5; FLC with pravastatin group: n = 5) were sacrificed by CO₂ asphyxia at day 7 and then the fungal burdens in liver and kidneys were determined, as described above.

Statistical analysis

The statistical significance of the survival data was ascertained by the Kaplan-Meier curves, and various groups were compared using the log rank test. Differences in the numbers of CFU per organs were analyzed by Student’s t-test. A probability value of < 0.05 was considered statistically significant.

Results

MICs of pravastatin and FLC against C. albicans

To test the antifungal activity of pravastatin, we determined MICs of selected strains of C. albicans to pravastatin in RPMI and YM broth [18]. In RPMI medium, pravastatin did not display anti-Candida activity, with MICs for nine strains of C. albicans to pravastatin found to be 1.024 μg/ml or above. Pravastatin displayed greater activity (MIC of 256 μg/ml) when YM broth was used in the MIC assay. Interestingly, a clear contrast was shown in the MICs to FLC between RPMI and YM broth. MICs of C. albicans against FLC were 0.125–0.25 μg/ml in RPMI broth, whereas those in YM broth were ≥ 16 μg/ml. These results suggest that MICs of pravastatin or FLC against C. albicans are significantly influenced by the medium used in studies. Notably, opposing results were observed for these two compounds. To clarify the mode of action against C. albicans, a growth inhibition study of pravastatin in YM broth was conducted. Pravastatin showed a dose-dependent growth inhibition, but no killing effects in YM broth during 24 h of incubation (static effect) (Fig. 2).

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Pravastatin and fluconazole have synergistic effect in vitro

The synergistic effects of pravastatin with FLC were assessed by checkerboard assays with *C. albicans* SC5314, ATCC 90028, and 06901B, as described in Materials and methods. The FICIs were calculated to be equal or below 0.5 in both YM broth and RPMI, suggesting pravastatin synergism with FLC in these three strains.

**Effects of pravastatin on farnesol production**

Farnesol was first discovered in a culture supernatant of *C. albicans*, and then characterized to have activity to suppress filamentation of this organism [20]. We started our studies by examining the effect of pravastatin on hyphae-suppressing activity in culture supernatants. As expected, addition of control culture supernatant (no pravastatin) suppressed hyphae formation from 66–26%.

In contrast, the culture supernatants prepared in the presence of pravastatin restored hyphae formation in a pravastatin-concentration dependent manner (Fig. 3A). These results suggested that incubation with pravastatin inhibited production of hyphae-suppressing molecules, presumably including farnesol, in the culture supernatant.

Next, we used TLC to directly assess the production and suppression of farnesol synthesis. Briefly, *C. albicans* was incubated with or without pravastatin (64 μg/ml), and then farnesol was extracted from the culture supernatant by ethyl acetate. Using TLC, farnesol production was confirmed in the control (no pravastatin) supernatant. The intensity of the corresponding spot in the culture supernatant was reduced when *C. albicans* was incubated with pravastatin (Fig. 3B). These results suggested that pravastatin at sub-MIC inhibited the synthesis of farnesol in *C. albicans*.

**Fig. 2** Growth inhibition in the presence of pravastatin. *Candida albicans* 06901B was suspended at a cell density of $1 \times 10^3$ cells per ml in YM broth containing the indicated concentration of pravastatin. Samples were incubated at 35°C on a shaker at 160 rpm. Pravastatin concentrations (in μg/ml) were 0 (filled circles), 128 (open circles), 256 (filled squares), 512 (open squares), and 1024 (filled triangles).

**Fig. 3** Effects of pravastatin on farnesol production. (A) *Candida albicans* 06901B was incubated in YM broth containing the indicated concentration of pravastatin. After 24 h of growth, the culture supernatants were collected and used for hyphal formation assay. Control solutions were prepared as YM broth containing the corresponding concentrations of pravastatin, but no *C. albicans*. Open squares: control solution; filled squares: culture supernatant. (B) *C. albicans* 06901B was incubated with or without pravastatin (64 μg/ml), and then the presence of farnesol was detected by TLC, as described in the materials and methods. Spots were quantified using ImageJ software.
Effects of pravastatin on fungal burdens and survival in systemic *C. albicans* infections

Finally, we examined the efficacy of pravastatin in treating systemic *C. albicans* infections. *C. albicans* (5 × 10⁴ CFU/mouse) was inoculated IV on day 0. Pravastatin (50 mg/kg) or control saline was administered IP once a day for 5 days, starting on the day of infection. Control (saline-treated) mice started to die from day 10, and all control mice died by day 13. In contrast, pravastatin-treated mice started to die from day 12, and 25% remained alive at the end of experiment (day 21; *P* = 0.03) (Fig. 4A). On day 12, a cohort of saline- or pravastatin-treated mice were sacrificed and the fungal burdens in liver and kidneys were determined. As shown in Fig. 4B, pravastatin-treated mice demonstrated significantly lower fungal loads compared to the control group (*P* = 0.01).

Next, combination effects of pravastatin (50 mg/kg) and FLC (4 mg/kg) were analyzed in the survival and fungal burdens using the same model. Mice were infected with higher inoculum doses (5 × 10⁵ CFU/mouse) than those used in pravastatin monotherapy (5 × 10⁴ CFU/mouse), and then the IP treatment (FLC alone or FLC + pravastatin combination) was administered once daily for 5 days starting 5 days after infection. Mice started to die from day 6 in both groups. A trend of delay of death was observed in the pravastatin combination group through the experimental period, although overall survival did not differ significantly between the two groups (*P* = 0.11) (Fig. 4D).
day 7, a cohort of FLC- or combination therapy-treated mice were sacrificed and the fungal burdens were determined. The fungal numbers in liver and kidneys were significantly lower in the pravastatin combination group ($P = 0.04$ and $0.03$, respectively) (Fig. 4E and 4F). Altogether, these data suggested that pravastatin treatment, alone or in combination with FLC, may be associated with survival benefits and the lowering of fungal burdens in systemic C. albicans infections.

**Discussion**

The present study demonstrates for the first time (to our knowledge) that pravastatin can improve survival and reduce fungal burden in a model of systemic C. albicans infection. In addition, pravastatin at sub-MIC was shown to suppress farnesol production in vitro, which may explain, at least in part, the in vivo efficacy of pravastatin. These data are consistent with previous reports that certain statins may possess anti-fungal activity in certain experimental conditions [9–13], and further suggest the potential of statins as virulence factor-suppressing agents that might be used as supportive anti-fungal therapies in C. albicans infections.

In vitro, pravastatin showed weak growth-suppressing activity against C. albicans, although this activity depended on the medium used for the determination of MICs. Although the exact mechanism of medium-dependent MIC changes remains unclear, a similar phenomenon was reported previously for FLC and lovastatin in RPMI and YEPD media [12]. On the other hand, the synergistic effects of FLC and pravastatin were consistently observed in both YM broth and RPMI in all three strains examined, in contrast to the lack of synergy reported by Nash et al. [21]. We note that the present study tested higher pravastatin concentrations (ranging up to 1,024 μg/ml) than those of Nash et al. (up to 1 μg/ml). We adopted these higher concentrations of pravastatin because we focused on the effect of inhibiting the fungal HMG-CoA reductase, not on a secondary effect of treatment for hyperlipidemia. This distinction is an example of the complexity and difficulty of assessing the in vitro antifungal activities of compounds. Numerous factors (e.g., pH, glucose and carbon dioxide levels, nitrogen source, and the presence of transition metals) will differ between in vitro and in vivo tests of antifungal activity. Moreover, in vivo efficacy may be further influenced by pharmacokinetic-pharmacodynamics parameters of compounds and host-derived serum and cellular factors.

Production of farnesol by C. albicans is the first quorum-sensing system discovered in a eukaryote [20]. Since farnesol is produced by an alternative pathway, diverging from the mevalonate pathway production of sterol intermediate farnesyl pyrophosphate, it is reasonable to speculate that HMG-CoA reductase inhibition may suppress production of farnesol. It was reported that farnesol inhibits the yeast-to-hyphae transition and biofilm formation [20,22,23]. As expected, hyphal formation was inhibited when the supernatant of spent medium of C. albicans culture was added in the assay. That inhibition was canceled if the spent medium was isolated from cultures grown in the presence of pravastatin. Finally, we demonstrated (by TLC) that farnesol production is reduced in the presence of pravastatin. It is likely that pravastatin is suppressing the production of hyphae-inhibiting factor in C. albicans. Although the factor(s) responsible for the morphological alterations in this assay remains undefined, the present study suggests that statins may impair the synthesis of HMG-CoA reductase-associated fungal products, including farnesol.

Navarathna et al. reported that fluconazole treated C. albicans had increased farnesol production [24]. It is possible that combination of pravastatin and fluconazole cancelled increase of farnesol production by fluconazole treatment, and that may be one of the reasons for the combination effect. For future study, more detailed analyses are needed to confirm the effect of HMG-CoA reductase inhibitors on farnesol production.

Our data demonstrate survival benefits and reduction of fungal burdens when pravastatin is administered systemically in a Candida mouse infection model. In separate studies (data not shown), we have observed significant improvement in survival in the same model when pravastatin is administered orally (PO). Komai et al. have reported that maximum plasma concentrations of pravastatin in rats, dogs, and monkeys were 0.75, 4.6 and 0.69 μg/ml, respectively, following administration at 20 mg/kg. PO [25]. Komai et al. also examined the tissue distribution of pravastatin in rats, in which higher levels were detected in the liver (11.45 μg/g) than in the kidney (2.00 μg/g) at 1 h after PO administration [25]. These results prompted the use of the liver to evaluate fungal burden in the present study. In human serum, lower concentrations of pravastatin (Cmax: 45–55 ng/ml) were reported following PO administration of 40 mg pravastatin [26]. Thus, the tissue concentrations of pravastatin in current clinical use may be far below the concentrations tested (in vitro and in vivo) in the present study. Further investigations, including the suppression of farnesol production in vivo, will be required to assess the potential therapeutic effects of pravastatin on systemic infections.

Statin research has focused recently on the potential of immunomodulatory effects of this class of compounds. Several investigators have reported beneficial activities of certain statins in sepsis patients [27,28]. Although the exact mechanisms of action are still obscure, multiple steps and factors, such as cell signaling, suppression of cytokine production, induction of heme oxygenase, and direct alteration of...
of leucocyte-endothelial cell interaction, were reported. In this context, we have examined the effects of pravastatin treatment on the levels of pro-inflammatory cytokines TNF-α and IL-6 in our systemic C. albicans infection model, but found no difference between control and pravastatin groups on days 1, 4, and 7 after infection (data not shown). Similar in vitro findings have been reported, in which the production of different amounts of farnesol by mutant strains of C. albicans had no influence on TNF-α and IL-6 secretion of macrophages. Therefore, pravastatin treatment might not exert an influence on pro-inflammatory cytokine secretion despite reduced farnesol production by C. albicans in systemically infected mice [29].

At least seven drugs in the statin class are now approved for clinical use for the treatment of hyperlipidemia. Lovastatin, pravastatin, and simvastatin are fungal-derived inhibitors of HMG-CoA, whereas atorvastatin, fluvastatin, rosuvastatin, and pitavastatin are fully synthetic compounds. It may be important to perform screening experiments for these statins and related analogues for their antifungal and anti-virulence activities, in addition to their immunomodulatory potentials. Given that the incidences of Candida infections continue to increase and significant numbers of cases are at risk for morbidity and mortality, the development of novel anti-fungal agents, including statin-derived or HMG-CoA reductase inhibitor-associated compounds, warrants future investigation.

Acknowledgements

We thank Yoshitugu Miyazaki for the kind gift of C. albicans SC5314, and Yuichi Kohyama and Akira Ikezaki for technical assistance. We express our deep appreciation to Tse-Hsien Koh for his critical comments and careful reviewing of the manuscript.

Declaration of interest: The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 28 September 2011


