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ARTICLES

Simvastatin Inhibits *Candida albicans* Biofilm *In Vitro*

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ABSTRACT: By inhibiting the conversion of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) to mevalonate, statins impair cholesterol metabolism in humans. We reasoned that statins might similarly interfere with the biosynthesis of ergosterol, the major sterol of the yeast cell membrane. As assessed by spectrophotometric and microscopic analysis, significant inhibition of biofilm production was noted after 16-h incubation with 1, 2.5, and 5 μM simvastatin, concentrations that did not affect growth, adhesion, or hyphal formation by *C. albicans in vitro*. Higher concentrations (10, 20, and 25 μM simvastatin) inhibited biofilm by >90% but also impaired growth. Addition of exogenous ergosterol (90 μM) overcame the effects of 1 and 2.5 μM simvastatin, suggesting that at least one mechanism of inhibition is interference with ergosterol biosynthesis. Clinical isolates from blood, skin, and mucosal surfaces produced biofilms; biofilms from bloodstream isolates were similarly inhibited by simvastatin. In the absence of fungicidal activity, simvastatin's interruption of a critical step in an essential metabolic pathway, highly conserved from yeast to man, has unexpected effects on biofilm production by a eukaryotic pathogen. **(*Pediatr Res* 66: 600–604, 2009)**

Candidemia is the fourth most common cause of bloodstream infection in hospitalized patients, with *Candida albicans* predominating (1). Hosts at greatest risk include burn patients, postpump cardiac surgery patients, neutropenic oncology patients, premature newborns, and patients recovering from abdominal surgery. In these groups, the incidence of candidemia ranges from 0.7 to 1.6 cases per 1000 discharges (2).

Age also exerts a profound effect, with the incidence in neonates (1.6 per 1000) about 8-fold greater than in adults (3). Premature newborns have significant higher risk, with an infection rate rising from 10 per 1000 in infants weighing 1001 to 1500 g to 75 per 1000 in infants ≤800 g (4). In all of these high-risk groups, mortality exceeds 25% in many studies (5–9). A common risk factor is the presence of central venous catheters (4,10) that serve as a site for the development of biofilm, composed of layers of yeast cells, hyphae, and a profuse exopolysaccharide matrix that renders the biofilm impenetrable to antibiotics and host defenses (11–13).

Clinical studies (14–17) and national guidelines (18,19) have emphasized that treatment of candidemia in those patients with a central venous catheter in place requires removal or replacement of the catheter, in addition to antifungal therapy, although catheter removal may disrupt the delivery of lifesaving chemotherapy, parenteral nutrition, antibiotics, or blood products. Although prophylaxis with fluconazole has proven effective in the prevention of *Candida* colonization in premature newborns as well as pediatric and adult oncology patients, the effect on mortality ascribable to candidemia has been variable (20–22). Thus, additional strategies for the prevention or impairment of biofilm production by *C. albicans* could be helpful.

Recently, our laboratory characterized two mitochondrial mutants in which the generation of acetyl CoA is impaired (23,24). Deletion of both copies of the gene encoding either complex I of the electron transport chain (*NDH51*) or pyruvate dehydrogenase complex protein X (*PDX1*) blocks the generation of acetyl CoA. Although we originally reported that these mutants displayed a wild-type phenotype with regard to growth, replication or hyphal formation in rich medium (23,24), we subsequently found that biofilm formation, as indicated by safranin staining of exopolysaccharide matrix, was significantly impaired in both mutants (Fig. 1) (25–27).

We therefore hypothesized that other inhibitors of acetyl CoA metabolism, such as the statins, might impair biofilm formation in *C. albicans* even without concomitant fungicidal effects. In higher eukaryotes, statins reversibly and competitively inhibit hepatic cholesterol synthesis from acetyl CoA by inhibiting 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which converts HMG-CoA to mevalonate (28,29). In more primitive eukaryotes such as *Candida albicans*, comparable steps in this essential pathway are highly conserved, but the final product is ergosterol, the major sterol of the yeast cell membrane (30). Therefore, we designed experiments to determine whether targeting an essential metabolic pathway in *C. albicans* could exert an inhibitory effect on production of biofilm by *C. albicans in vitro* in the absence of fungicidal activity.

METHODS

Chemicals. Simvastatin (Sigma Chemical Co.-Aldrich), provided in the form of a lactone prodrug, was activated by solubilization in 15% (vol/vol)

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Abbreviations: YPD, yeast peptone dextrose

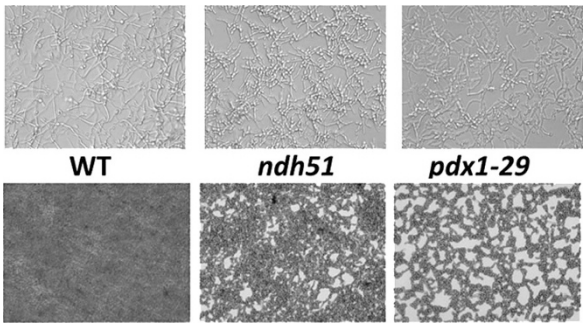

Figure 1. Photos in *top row* illustrate hyphal formation in rich radium for wild-type (WT) strain and isogenic mutants deleted in the 51 kDa subunit of nicotinamide adenine dinucleotide dehydrogenase complex I (*ndh51*) or in pyruvate dehydrogenase complex protein X (*pdx1-29*); photos in *bottom row* show safranin staining of corresponding mature biofilm at 16 h.

ethanol and 0.25% (wt/vol) NaOH at 60°C for 1 h (31). Stock solutions of hydrolyzed simvastatin were stored at −20°C at 20 mg/mL (48 mM). Ergosterol (Fluka, Switzerland) was dissolved at 50 mg/mL (126 mM) in Tween 80:ethanol (1:1) at 60°C for 10 min. Filipin (Polysciences Inc.) was reconstituted in DMSO at 50 mg/mL, as described (32–34). Safranin (Acros Organics) was solubilized in H_2O at a working concentration of 0.1% (wt/vol) (25,26).

Growth of* C. albicans *strains. *Candida albicans* laboratory strain BWP17wt and the isogenic mutants deleted for both alleles of *NDH51* (*ndh51*) and *PDX1* (*pdx1–29*) have been previously described (23,24), as have clinical isolates from blood, skin, and mucosal surfaces (35). All strains were maintained at −80°C in 20% glycerol. Working cultures were plated on YPD (yeast/peptone/dextrose) agar plates (1% yeast extract, 2% Bacto-peptone, 100 mM dextrose, 1.5% Bacto-agar) at 30°C for 48 h. A single colony of the desired strain was inoculated into 2.5 mL YPD medium and incubated overnight at 30°C with shaking.

Biofilm assays. The biofilm assay has been previously published (25–27). Overnight cultures were diluted to a concentration of 2.5×10^5 colony-forming units/mL in YPD (OD_{600}). Yeast cells (100 μL) were inoculated into each well of a 96-well flat-bottom polystyrene culture plate (Corning 3595) and incubated at 37°C for 3, 6, or 16 h in a humidified chamber. Simvastatin was added at concentrations ranging from 1 to 25 μM, ergosterol at 90 μM and filipin at 50 μg/mL, as indicated. Cells not treated with simvastatin were incubated with solvent (0.25% NaOH/15% ethanol) equivalently diluted in YPD. Each reaction was performed in triplicate wells. For quantitation, medium was removed at the indicated time point, and biofilms were washed twice with PBS, stained with 100 μL safranin for 10 min at room temperature, washed 3× with distilled H_2O, and scanned at OD_{405} with a Spectra Max 190 plate reader (Molecular Devices, Sunnyvale, CA) using Soft max Pro 4.3.1 software. In certain experiments, filipin (50 μg/mL) was added to planktonic cells after 16-h incubation with simvastatin (5 μM) ± ergosterol (90 μM); cells were observed immediately using a DAPI filter at 358 nm excitation and 461 nm emission wavelength. For all biofilms, microscopic analysis of staining with safranin or filipin was performed with an Olympus IX70 fluorescent microscope using a 40× objective lens. Digital images were captured with an Optronics Engineering Camera (DEI-750) and Image-Pro Plus 5.0 software.

Statistical analysis. In the dose-response experiments, nine separate experiments were done across the entire range of simvastatin concentrations (0–25 μM). In a *post hoc* analysis, the Wilcoxon signed ranks test was used for paired comparison (total comparisons = 6), and the critical p value was adjusted with the Bonferroni correction (0.05/6 = 0.008).

For the experiments examining the effects of exogenous ergosterol in reconstituting biofilm formation, the Wilcoxon signed ranks test was used to test whether the addition of ergosterol resulted in the difference of the observed production of biofilm as measured by spectrophotometry. Again, the Bonferroni correction was applied to adjust for the three pair-wise comparisons (1, 2.5, and 5 μM simvastatin), which resulted in the critical p value of 0.017.

RESULTS

Assessment of biofilm production. Spectrophotometric analysis of safranin staining was used to quantitate the effects of simvastatin (1–25 μM) on mature biofilms produced by *C. albicans* (Fig. 2). After 16-h incubation, a concentration of 1 μM simvastatin reduced biofilm formation by 19%. However, concentrations of 2.5 to 25 μM simvastatin reduced biofilm formation by ≥87%. These reductions were statistically significant ($p = 0.007$). Quantitative cultures of organisms in biofilms showed that simvastatin concentrations ≤5 μM were not candidacidal; simvastatin concentrations ≥10 μM led to a 1-log kill.

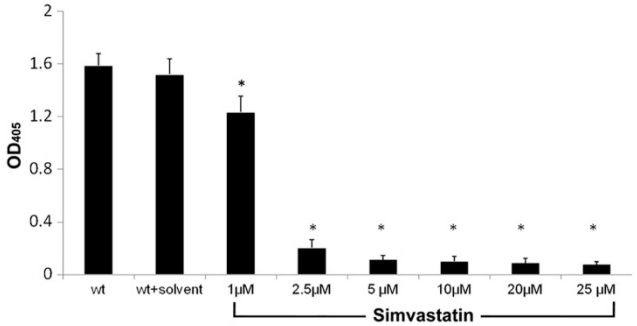

Figure 2. Assessment of mature biofilms at 16 h in the absence or presence of the indicated concentrations of simvastatin. Spectrophotometric quantitation of safranin staining was performed at OD_{405}. Bars represent mean ± SD for $n = 9$ experiments with the indicated concentration of simvastatin. *p = 0.0007 for 1.0 to 25 μM simvastatin compared with the wild-type strain + solvent.

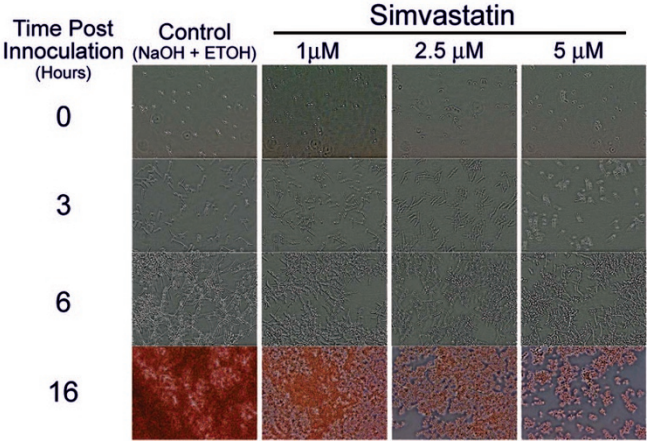

Figure 3. Time- and dose-dependent microscopy of biofilm formation in the absence or presence of the indicated concentrations of simvastatin.

To determine whether early changes in adhesion or hyphal formation were responsible for simvastatin's effects on mature biofilm, biofilms were assessed microscopically after 0, 3, or 6 h treatment with solvent (control) or the indicated concentration of simvastatin (Fig. 3). Concentrations between 1 and 5 μM were chosen for detailed analysis because of the results in Figure 2 and because these concentrations are ~20- to 100-fold less than those previously reported to be cidal for *C. albicans* (36). At time 0 and after 3 h, similar numbers of cells adhered in the presence of 0, 1, 2.5, or 5 μM simvastatin, whereas at 6 h, both untreated and simvastatin-treated cells produced hyphae. However, as assessed by safranin staining at 16 h, *C. albicans* cells treated with 2.5 or 5 μM simvastatin showed marked impairment of biofilm production, compared with cells treated with solvent alone.

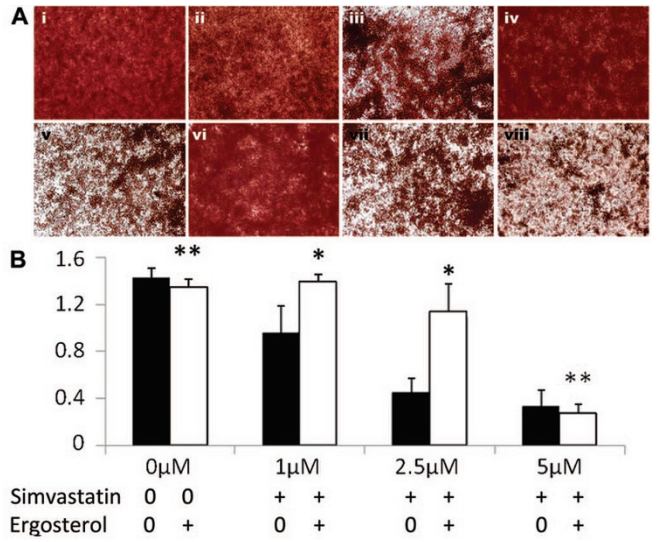

Figure 4. *A*. Microscopic analysis of reconstitution of simvastatin-inhibited biofilm formation by ergosterol. Panels *i*, *iii*, *v*, and *vii* are biofilms treated with NaOH/ethanol solvent (*i*) or 1 μM (*iii*), 2.5 μM (*v*), or 5 μM (*vii*) simvastatin, respectively. Panels *ii*, *iv*, *vi*, and *viii* received 90 μM ergosterol in addition to solvent and simvastatin. *B*. Spectrophotometric analysis of reconstitution of simvastatin-inhibited biofilm formation by ergosterol. ■: mean ± SD for $n = 7$ experiments with the indicated concentration of simvastatin. □: mean ± SD for $n = 7$ experiments with the indicated concentration of simvastatin plus 90 μM ergosterol. *$p = 0.018$ for the indicated pairs, **not significant.

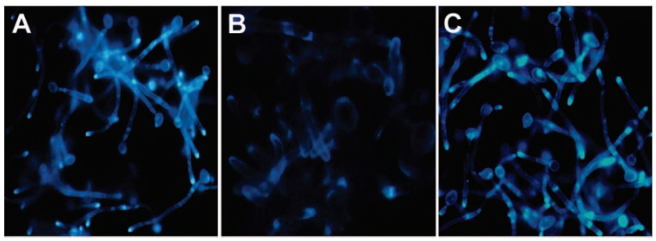

Figure 5. Filipin staining of planktonic *Candida albicans* cells in the absence (*A*), and presence of 5 μM simvastatin (*B*), or 5 μM simvastatin + 90 μM ergosterol (*C*).

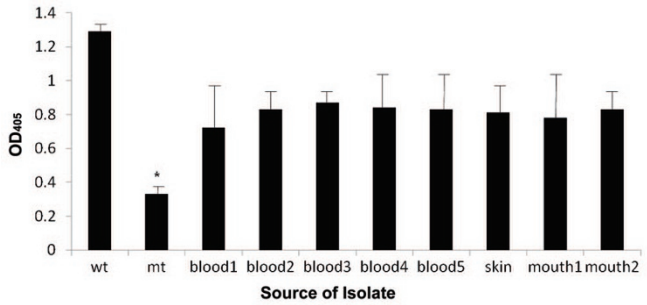

Figure 6. Biofilm production by the wild-type strain BWP17wt, the mutant *pdx1–29*, and eight clinical isolates. Results are the mean ± SD for $n = 3$ experiments. *$p = 0.01$.

Mechanism of inhibition. Because concentrations of simvastatin that inhibited biofilm production (≥1μM) did not seem to affect growth, adherence, or hyphal formation of *C. albicans* (Fig. 2), we used microscopy to assess the ability of exogenous ergosterol to overcome the inhibitory effects of simvastatin (Fig. 4*A*). Ergosterol did not augment safranin staining of 16 h biofilms formed by control organisms treated with solvent (NaOH/ethanol). In the presence of 1 or 2.5 μM simvastatin, production of *C. albicans* biofilm was visually impaired; however, addition of ergosterol restored safranin staining. In the presence of 5 μM simvastatin, the addition of ergosterol was not sufficient to restore safranin staining, suggesting that other statin-dependent or -independent processes may be compromised under these conditions.

These results were substantiated in a spectrophotometric assay (Fig. 4*B*). In the absence of simvastatin, addition of ergosterol did not increase safranin staining of mature (16 h) biofilms. Incubation with 1 μM simvastatin led to a decrease in staining, which improved with the addition of ergosterol ($p = 0.018$). The same result was true for 2.5 μM simvastatin: the addition of ergosterol restored safranin staining ($p = 0.018$). However, in the presence of 5 μM simvastatin, ergosterol proved ineffective, again indicating that simvastatin's inhibitory effects may not be confined to ergosterol biosynthesis.

The effects of simvastatin on ergosterol production were corroborated by staining with filipin, a dye that is specifically taken up by ergosterol (33,34). Detection of ergosterol in the cell wall and hyphae of *C. albicans* cells was quite distinct in the absence of simvastatin, but in the presence of simvastatin, ergosterol was spotty and disordered (Fig. 5). The addition of ergosterol to the simvastatin incubation mixture restored filipin staining in planktonic cells, even though biofilms treated with 5 μM simvastatin were not rescued by ergosterol (Fig. 4*A* and *B*). These results provide additional confirmation that at least one mechanism by which simvastatin inhibits *C. albicans* relates to interference with ergosterol synthesis, which may be more susceptible to simvastatin in organisms composing a biofilm, as opposed to planktonic cells.

Corroboration with clinical isolates. Biofilm production by clinical isolates from blood ($n = 5$), skin ($n = 2$), and mucosal surfaces ($n = 2$) was assessed by safranin staining in the absence or presence of 5 μM simvastatin (Fig. 6). Laboratory strain BWP17wt proved to be the most vigorous biofilm producer *in vitro*, whereas the biofilm produced by mutant *pdx1–29* was markedly decreased as previously observed. All clinical isolates produced biofilms, but safranin staining was less intense than with the laboratory strain. By spectrophotometric assay, biofilm formation by four blood isolates from patients with catheter-associated candidemia was inhibited by 5 μM simvastatin, with decreases in OD_{405} ranging from 53 to 59%. The effects of simvastatin on biofilms formed by skin and mucosal isolates were not tested.

DISCUSSION

Results of these *in vitro* studies show that noncandidacidal concentrations of simvastatin (1, 2.5, or 5 μM) inhibit biofilm production *in vitro* by laboratory and clinical isolates of *C. albicans* in both microscopic and spectrophotometric assays. Although microscopic and spectrophotometric assays showed a modest inhibitory effect of 1 μM simvastatin, repeated analysis in a spectrophotometric assay showed statistically significant inhibition ≥87% at concentrations of 2.5 μM and above. At concentrations ≤5 μM, simvastatin does not inhibit

growth of *C. albicans*, and neither adhesion at 3 h nor hyphal production at 6 h is impaired in organisms incubated with 1, 2.5, or 5 μM simvastatin (Fig. 3).

The ability of exogenous ergosterol to restore biofilm formation after treatment with 1 or 2.5 μM simvastatin (Fig. 4*A* and *B*) indicates that at least one inhibitory mechanism is interference with ergosterol synthesis. Although genes involved in the ergosterol pathway are known to contribute to biofilm formation (37,38), the failure of ergosterol to restore biofilms treated with 5 μM simvastatin suggests that concentrations $\geq$5 μM may impair other metabolic processes as well, such as statin-dependent geranylgeranylation, farnesylation, or production of exopolysaccharide. Interestingly, as the simvastatin concentration exceeded 5 μM, quantitative cultures of biofilms yielded increasing numbers of petite mutants, which represent organisms that have lost mitochondrial DNA. Petite mutants have been reported in *Candida glabrata* with simvastatin concentrations of 60 μM (39).

Although enzyme conformation and location of the active site lysine differ between Class II (bacterial) and Class I (eukaryotic) HMG CoA reductases, the isoprenoid pathway is essential for many microbes (40–42). Although Class II enzymes are much less sensitive to inhibition by statins, inhibition of bacterial or fungal growth by interference with the isoprenoid pathway is well described. An inhibitor of squalene synthetase, an intermediate step in the cholesterol pathway, blocked staphyloxanthin production and attendant virulence in *Staphylococcus aureus* (43). Lovastatin-mediated growth inhibition has been reported for several pathogenic and nonpathogenic yeasts and one mold (31,39,44). For example, 100 μM simvastatin or atorvastatin inhibited growth of four *Candida* species (*C. albicans*, *tropicalis*, *glabrata*, and *parapsilosis*) as well as *Aspergillus fumigatus* (36).

Our studies are the first to examine the effect of statins on *C. albicans* biofilm. The lowest concentration of simvastatin that impaired biofilm formation in our studies (1 μM) is 10-fold less than the concentration that inhibited growth of *C. albicans* in our experiments and ~60 to 100-fold less than the candidacidal concentration reported in previous work (36). Nevertheless, 1 μM simvastatin is still ~30-fold higher than the plasma level achieved after standard oral dosing in humans (28). Although pharmacoepidemiologic studies have found no increased risk for fetal anomalies in pregnant women taking statins (45), reformulating simvastatin as a coating for catheter lumens may be a more feasible approach, as has been done with other substances such as EDTA or amphotericin, where oral dosage is impractical (46,47).

Although these studies report an inhibitory effect of statins on biofilm production by *C. albicans* laboratory and clinical isolates *in vitro*, *in vivo* studies in an animal model of catheter-associated bloodstream infection will be required to confirm clinical relevance. It is certainly possible that other statins or pharmacologic analogs will prove even more effective than simvastatin, either *in vitro* or *in vivo*. Indeed, a small molecule screen to identify potentially effective compounds is underway with our collaborators. The studies reported here represent a first step in demonstrating that interference with an essential pathway of lipid synthesis in *C. albicans* may have unexpected and potentially clinically relevant effects on biofilm formation.

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