Minocycline Inhibits *Candida albicans* Budded-to-Hyphal-Form Transition and Biofilm Formation

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**SUMMARY:** *Candida albicans* frequently causes bloodstream infections; its budded-to-hyphal-form transition (BHT) and biofilm formation are major contributors to virulence. During an analysis of antibacterial compounds that inhibit *C. albicans* BHT, we found that the tetracycline derivative minocycline inhibited BHT and subsequent biofilm formation. Minocycline decreased expression of hypha-specific genes *HWPI* and *ECEI*, and adhesion factor gene *ALS3* of *C. albicans*. In addition, minocycline decreased cell surface hydrophobicity and the extracellular β-glucan level in biofilms. Minocycline has been widely used for catheter antibiotic lock therapy to prevent bacterial infection; this compound may also be prophylactically effective against *Candida* infection.

**INTRODUCTION**

The opportunistic fungus *Candida albicans* is a common cause of nosocomial bloodstream infections; this microorganism can form a biofilm by adhering to the surface of medical devices (1). Annually, more than 10 million patients require treatment with various medical devices, and device-associated candidemia can have mortality rates of up to 30% (2,3). *C. albicans* biofilms have complex structure consisting of yeast-form cells and hyphal cells embedded in a matrix of extracellular polymeric substances (4). These biofilms are relatively resistant to antimicrobial agents, and surgical removal of an infected device is often needed to treat a biofilm-associated infection (2). The minimum inhibitory concentration of the azole agent fluconazole used to treat infection caused by a *C. albicans* biofilm is approximately 1,000-fold greater than that of planktonic cells (5). Therefore, inhibition of biofilm formation is clinically significant. Biofilm formation by *C. albicans* involves a budded-to-hyphal-form transition (BHT). Toenjes et al. found that 23 small molecules inhibit *C. albicans* BHT (6,7) and subsequently identified 3 compounds that are effective against *C. albicans* biofilm formation: buhytirin A, ETYA, and CGP-37157 (8).

Here, when evaluating compounds inhibiting the BHT of and biofilm formation by *C. albicans*, we found that the antibacterial agent minocycline was active against both the hyphal formation and biofilm formation. Minocycline, a derivative of tetracycline, is a broad-spectrum antimicrobial agent inhibiting bacterial protein synthesis; the drug has been used for coating catheters in an effort to prevent device-related infections (9).

In the present study, we explore the activity of minocycline against *C. albicans* BHT and biofilm formation and hypothesize that the observed antibiofilm effect is mediated by decreased production of adhesins and extracellular matrix components by yeast cells.

**MATERIALS AND METHODS**

**Strains and antibacterial agents:** *C. albicans* SC5314 was routinely grown in Sabouraud dextrose agar plates at 27°C. Five tetracycline derivatives were examined in this study: minocycline, tetracycline, oxytetracycline, and demethylchlortetracycline were obtained from Wako Pure Chemicals (Osaka, Japan) and doxycycline from Sigma-Aldrich (St. Louis, MO, USA). All the derivatives were dissolved in dimethyl sulfoxide (DMSO) and stored at –30°C until use.

**Inhibition of the bud-to-hypha transition:** The spider medium, *N*-acetylglucosamine (GlcNAc) medium, and Lee’s medium were used for hyphal induction (10,11). *C. albicans* cells (10^6/mL) were added to each medium together with final concentrations 10 μg/mL of each test compound, in 96-well plates. The plates were incubated statically at 37°C for 48 h, and hyphal formation was evaluated microscopically.

**Biofilm formation:** Biofilms were formed by cells growing in flat-bottomed, 96-well microtiter plates. Minocycline was added to final concentrations of 0, 1, 5, and 10 μg/mL. Standardized cell suspensions (inocula: 10^6 cells/mL in the RPMI 1640 medium) were seeded in each well. The plates were incubated statically at 37°C. After 48 h, planktonic cells were removed, and the wells were washed with phosphate buffered saline (PBS). Semiquantitative evaluation of biofilm formation was performed by the XTT (2,3-Bis-(2-methoxy-4-nitro-5-sulphonyl)-2H-tetrazolium-5-carboxanilide) reduction assay (5).

**Quantitative reverse transcription PCR (RT-qPCR):** *C. albicans* cells (10^6/mL) were added to the RPMI 1640 medium with minocycline. The culture dishes were incubated statically at 37°C for 48 h. After a wash with PBS, *C. albicans* cells were collected, and RNA was extracted. cDNA was synthesized using a
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F, forward primer; R, reverse primer.

Results

Inhibitory effects of tetracycline derivatives on C. albicans BHT and biofilm formation: Three hypha-inducing media—Spider, N-acetylglucosamine, and Lee’s media—were used to test the inhibitory effects of tetracycline derivatives. At concentrations of 10 μg/mL of each derivative, no inhibitory effects were observed in the presence of tetracycline, doxycycline, oxytetracycline, or demethylchlorotetracycline, in any of the 3 media. Only minocycline inhibited the BHT and did so in all 3 media at a concentration of 10 μg/mL (Fig. 1). In the microdilution assay, 128 μg/mL minocycline did not inhibit the growth of C. albicans at an inoculum of 10⁶ CFU/mL, which is equivalent to that used in the present.

Cell surface hydrophobicity assay: Microbial cell surface hydrophobicity (CSH) was measured using a water–hydrocarbon two-phase assay (16). Briefly, C. albicans biofilms were removed from the surfaces of cell culture dishes and resuspended in the YPD medium (1.0% yeast extract, 2.0% peptone, and 2.0% dextrose [all w/v]) to prepare cell suspensions (absorbance at 600 nm [A₆₀₀] = 1.0). Aliquots (1.2 mL) of these suspensions were pipetted into sterile tubes, overlaid with 0.3 mL of octane, and vortexed for 3 min. After separation of the 2 phases, A₆₃₀ values of the aqueous phases were determined. The value of a sample not overlaid with octane served as the control. Relative hydrophobicity values were calculated as ([A₆₃₀ of control sample] - [A₆₃₀ of octane overlay])/[A₆₃₀ of control sample] × 100.

Soluble β-1,3 glucan assay: Biofilms were grown at 37°C in cell culture dishes with the RPMI 1640 medium in the presence of minocycline. Supernatants from the biofilm cultures were collected after 48-h incubation for glucan measurements, and viable cell numbers were determined by plate counting. The supernatants were centrifuged at 16,000 g for 5 min and stored at −30°C before glucan analysis. Glucan concentrations were determined using a Glucatell (1,3)-β-1,6-Glucan Detection Reagent Kit (Associates of Cape Cod, East Falmouth, MA, USA).

Fig. 1. Minocycline inhibits the C. albicans yeast-to-hypha transition. C. albicans was cultured in Spider, N-acetylglucosamine, and Lee’s hypha-inducing media, in the presence of 10 μg/mL of each tetracycline derivative, at 37°C. Cells were visualized under a light microscope at 48 h after inoculation.

Fig. 2. Minocycline inhibits C. albicans biofilm formation. Biofilm formation was evaluated using the XTT reduction assay; the results are presented as percentages of the control (no antibiotic). *P < 0.05, **P < 0.005, ***P < 0.001 compared with the control.

PrimeScript RT Master Mix (Takara, Shiga, Japan). RT-qPCR was conducted by means of the Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA); the primers are listed in Table 1.

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Therefore, the anti-BHT effect of minocycline at a concentration of 10 μg/mL is not attributed to the growth-inhibitory activity. Of the 5 compounds tested, only minocycline inhibited BHT; thus, the ability of this compound to inhibit biofilm formation was studied next. Biofilm formation was inhibited by minocycline in a concentration-dependent manner at levels ≥ 5 μg/mL (Fig. 2).

Gene expression in *C. albicans* biofilms in the presence of minocycline: Given that only minocycline clearly inhibited the BHT and biofilm formation, changes in the expression levels of genes known to affect hyphal formation, adhesion, and biofilm production were evaluated in the presence of minocycline by RT-qPCR (Fig. 3). Hypha-specific genes *HWP1* and *ECE1*, and the adhesion-related gene *ALS3* were downregulated by 10 μg/mL minocycline. The expression level of a biofilm-related gene *BCR1* and that of hypha-related transcription factor genes *EFG1*, *CPH1*, and *TEC1*, were also downregulated, while *NRG1* and *TUP1* were essentially unaffected by 10 μg/mL minocycline.

CSH of cells of *C. albicans* biofilms grown in the presence of minocycline: To evaluate fungal adhesion ability, we assessed the effect of minocycline on CSH, which has been reported to positively correlate with adhesion. Minocycline decreased biofilm CSH at 10 μg/mL (Fig. 4).

The effect of minocycline on the concentration of soluble β-1, 3-glucan from the biofilm extracellular matrix in the culture supernatant: The *C. albicans* matrix consists primarily of carbohydrates, with β-1,3-glucan being the principal component. We measured soluble β-1,3-glucan levels in biofilm culture supernatants exposed to various concentrations of minocycline. The β-glucan levels were standardized to the numbers of viable biofilm cells. The standard deviations of 3 independent experiments are shown. *P < 0.05 compared with the control.

**DISCUSSION**

Of the later discovered tetracycline derivatives, only minocycline inhibited both the BHT of and biofilm formation by *C. albicans*. The BHT is triggered by several external signals, including elevated temperature or pH, nitrogen and/or carbon starvation, and exposure to serum. Hypha-specific genes *HWP1* and *ECE1*, adhesion-related gene *ALS3* have been identified (17–21),
and the pathways controlling the expression of these genes have been well-studied. Each pathway culminates in the activation of different transcription factors, inducing expression of hypha-specific genes. The Cek1 MAPK signaling pathway activates the Cph1 transcription factor gene (22,23), whereas the cAMP-PKA signaling pathway activates the Efg1 transcription factor gene (24). Cph1 and Efg1 are induced in GlcNAc and Spider media, respectively (10,25,26). The Tec1 transcription factor is activated by growth in Lee’s medium (10,27,28).

The Tup1 BHT repressor is constitutively expressed but inhibits the BHT only in combination with 3 other negative regulators, Nrg1, Mig1, and Rfg1 (10,29–31) (Fig. 6). Bcr1 regulates the initiation of biofilm formation, and its expression level increases during the hyphal stage, although this protein is required for biofilm formation, it is not involved in the production of normal hyphae (19,32). Overexpression of HWP1, ALS3, and ECE1 enhances biofilm formation by a Δber1 mutant defective in normal biofilm formation (18,19). Here, at a concentration of 10 µg/mL, only minocycline inhibited both the BHT and biofilm formation in all the media tested (Fig. 1 and 2). RT-qPCR results indicated that hypha-, adhesion-, and biofilm-related genes (including HWP1, ALS3, ECE1, EFG1, CPH1, TEC1, and BCR1) were downregulated by minocycline (Fig. 3). Downregulation of HWP1, ALS3, ECE1, and BCR1 may inhibit the initiation of biofilm development. EFG1, CPH1, and TEC1 encode transcription factors active in the signal transduction pathway required for hyphal formation, as mentioned above. Downregulation of these genes indicates that the antibiofilm activity exerted by minocycline is mediated by the signal transduction pathway required for hyphal formation. Expression levels of adhesion-related genes are reduced by minocycline; however, many genes play complementary roles in adhesion and biofilm formation. Nobile et al. (33) found that both a Δhwp1 mutant and a Δals1/Δals3 double mutant are defective in biofilm formation; however, a mixture of the 2 mutant strains produces a biofilm equivalent to that of a wild-type strain. A positive correlation between CSH and the adhesion of C. albicans has been reported (34).

We evaluated the adhesion ability by measuring the CSH of biofilm cells after exposure to minocycline. CSH was reduced in the presence of minocycline (Fig. 4), suggesting that the compound inhibited biofilm formation by decreasing the adherence of C. albicans cells.

As a biofilm matures, extracellular matrix accumulates and contributes to maintenance of the biofilm as well as affords resistance to antimicrobial drugs. The C. albicans matrix is composed primarily of carbohydrates but also includes proteins, hexosamine, phosphorus, and uronic acid (35). Of the various carbohydrates present, the most abundant is β-1,3-glucan, which is a component of the cell wall of C. albicans and is secreted by biofilms grown in vitro. C. albicans biofilms grown in an in vivo model of catheter infection produce soluble β-1,3-glucan (36). Nobile et al. (37) have reported that a null mutant of zap1 (a negative matrix regulator) shows increased production of soluble β-1,3-glucan. The amounts of this substance in culture supernatants decreased in the presence of minocycline (Fig. 5).

Catheters coated with minocycline and rifampin have been used to reduce the risk of catheter colonization and infection (38). Although intended to counter bacterial infections, we found that these catheters (especially minocycline-coated ones) may be useful for prevention of catheter-associated C. albicans infections, in the present study.

Several inhibitors of hyphal formation and biofilm formation by C. albicans have already been reported, including buhytrin A, ETYA, and CGP-37157 (8). Nonetheless, their structures do not provide insights into their potential activity. Besides, there are no structure-activity relations between these compounds and minocycline; thus, the targets of these compounds may be different. Of the 5 tetracycline derivatives examined, only minocycline inhibited the BHT and biofilm formation.

Chemically, the basic skeleton of tetracycline antimicrobial agents is characterized by an A ring attached to an L-shaped in-planar BCD ring with several functional hydrophilic groups (39). The presence of Mg²⁺ and a formal sequence of polar functional groups at positions 1 and 10–12a, including a carbamoyl group at position 2, are necessary for binding to the ribosomal 30S subunit. The antimicrobial activities of tetracycline derivatives are based on these chemical characteristics. Tetracycline derivatives also affect mitochondrial ribosomes in eukaryotic cells (40). Nevertheless, mitochondrial ribosomes may not be a target in BHT and biofilm inhibition because only minocycline showed this effect. Although farnesol, a quorum-sensing molecule of C. albicans, affects BHT, its analogs do not have an equivalent activity, pointing to the existence of a farnesol-binding receptor protein (41). There may also be a minocycline-binding protein in C. albicans. Minocycline has a dimethylamino group at the position 7. This is a bulky group that can be either hydrophilic or hydrophobic. No detailed structure–activity correlations are yet available; however, the dimethylamino group may be required for inhibition of C. albicans BHT and biofilm formation.

In conclusion, minocycline inhibits both the BHT of and biofilm formation by C. albicans, possibly by reduc-
ing cellular adhesion via modulation of each BHT pathway. Additionally, the extracellular-matrix amount was reduced upon minocycline treatment. Further in vivo research is required for testing whether minocycline effectively prevents catheter-related *C. albicans* infections.

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**Conflict of interest** None to declare.

**REFERENCES**