Inhibitory Effect of Resveratrol on Candida albicans Biofilm Formation

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Abstract

Candida albicans is the primary candidiasis-causing fungal pathogen in humans, and one of its most important virulence factors is the ability to form biofilms. Moreover, these biofilms are often resistant to antifungal agents, so there is a need to develop alternative elimination strategies and therapeutic agents for such infections. The antifungal activity of resveratrol, a phytoalexin polyphenolic compound, impairs the morphological transition of C. albicans under various hypha-inducing conditions and inhibits growth of the yeast-form and mycelia. The purpose of this study was to investigate the effect of resveratrol against C. albicans biofilm formation. The developmental, sustained, and mature stages of biofilm formation were affected or inhibited by resveratrol. Exposure to resveratrol at the developmental stage inhibited growth of C. albicans in a dose-dependent manner. A >30% reduction was observed in sustained biofilm growth in the presence of 200 μg/ml resveratrol in comparison with in its absence. In terms of disruption of matured biofilm, 6.25–100 μg/ml resveratrol significantly reduced cell viability of C. albicans compared with in a control sample (p<0.05). The present results indicate that resveratrol has the potential to serve as an anti-Candida treatment and preventive tool which functions by inhibiting existing or under-forming C. albicans biofilms.

Key words: Candida albicans—Resveratrol (trans-3, 4’, 5-trihydroxy-trans-stilbene) — Dimorphism — Biofilm

Introduction

Colonization by the fungal pathogen Candida albicans is common in healthy individuals. It is an opportunistic pathogen which can give rise to both mucosal and systemic infections in immunocompromised hosts⁷. Dimorphism, the ability to switch from yeast
to its hyphal form, and biofilm formation are important virulence properties of *C. albicans*, both of which contribute to its ability to infect host organisms\(^{14,22}\). Biofilm formation by *C. albicans* is an important virulence attribute in the medical field, as these biofilms can develop on medical devices, enabling the spread of the microorganism into the bloodstream and to various organs of the human body. The process of *C. albicans* biofilm formation occurs in four distinct stages: (1) adherence of yeast-form cells to a surface to seed a biofilm; (2) initiation of biofilm formation, the adhered cells proliferating to form an anchoring basal layer; (3) maturation of the biofilm, the cells in the initiated biofilm continuing to proliferate and produce filaments, resulting in complex layers of polymorphic cells encased in an extracellular matrix; and (4) dispersion, yeast-form cells being released from the mature biofilm to seed new sites (Fig. 1)\(^{1,15,17,24}\). Biofilms protect microorganisms from host immune system defenses and are highly resistant to antifungal agents. Furthermore, they function as a protected reservoir to seed and disseminate the infection\(^{125}\). Thus, when *C. albicans* cells form biofilms, they show higher antifungal resistance than planktonic cells and develop pathogenicity and multidrug-resistant traits. This often leads to the failure of therapeutic strategies and makes it difficult to exterminate *C. albicans* biofilms by conventional drug treatments. Hence, the need to develop and discover new antifungal strategies and effective therapeutic agents.

Resveratrol (trans-3, 4’, 5-trihydroxy-trans-stilbene) is a phytoalexin polyphenolic compound produced by the innate host defense systems of plants\(^{2,11}\). Various pharmacological effects and biological activities of resveratrol and its molecular mechanisms have been reported\(^{6,20}\). An earlier study by the present group reported the antifungal activity of resveratrol against both yeast-form and mycelial growth, and showed that it impaired the morphological transition of *C. albicans* under various hypha-inducing conditions\(^{19}\). The present study was designed to gain a further understanding of the antifungal activity of resveratrol by elucidating its effects on the growth of *C. albicans* biofilm.

**Materials and Methods**

1. **Strain and media**

*Candida albicans* strain SC5314 was used in this study. *Candida albicans* yeast was routinely maintained on yeast extract peptone dextrose (YPD; 1% yeast extract, 2% peptone, and 2% glucose) agar plates and grown in YPD medium at 30°C\(^{8}\).

2. **Reagent**

Resveratrol was purchased from Sigma-Aldrich (St. Louis, MO, USA) and dissolved to a concentration of 50 mg/ml in ethanol as a stock solution.
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3. Biofilm formation
An earlier study by this group revealed that both yeast-form and mycelial growth of C. albicans were inhibited by resveratrol. Resveratrol at concentrations of 60–200 μg/ml and 40–200 μg/ml inhibited yeast and mycelial growth of C. albicans, respectively[19]. Therefore, in the present study, a concentration of 6.25–200 μg/ml resveratrol was adopted and added at a two-fold dilution to the media. Biofilm formation was evaluated in accordance with the modified protocol for the Candida biofilm microtiter assay described by Gulati et al[8]. Briefly, 1 × 10⁷ C. albicans yeast cells/ml in 200 μl RPMI-1640 medium were incubated in 96-well microplates at 37°C, with a wash step with phosphate-buffered saline (pH 7.2) to remove non- or weakly-adhered cells. To determine the effect of resveratrol at specific time points, various concentrations (6.25–200 μg/ml) were added at the adherence (0–90 min) and developmental (90 min–24 hr) stages, while normal medium only was used at the remaining stages of biofilm formation. For the sustained growth inhibition assay, resveratrol was added at both the 90-min adherence and 24-hr growth stages. After 24 hr, the biofilms formed were quantified by using the water-soluble tetrazolium salts (WST)-1 assay described below. Three independent experiments for each assay were carried out, and each experiment was performed in quadruplicate.

4. Biofilm disruption assay
Assays were performed in a 96-well format as previously described to assess the ability of resveratrol to disrupt an established mature biofilm[8,16]. The original medium was removed and the 24-hr-old biofilm exposed to various concentrations of resveratrol at 37°C for 24 hr in fresh medium. After incubation, biofilm cell viability was quantified with the WST-1 assay. Three independent experiments were carried out, and each was performed in quadruplicate.

5. WST-1 reduction assay
Candida albicans biofilm formation and cell viability were assessed using Cell Proliferation ReagentWST-1 (Roche Diagnostics, Mannheim, Germany). Colorimetric change was measured at 440 nm, which is based on the cleavage of the tetrazolium reagent to water-soluble formazan by cellular mitochondrial dehydrogenase. This method is considered suitable as a quantitative cell proliferation assay for Candida cells[3,10]. Briefly, 50 μl of the WST-1 mixture was added to each well and the plates then incubated at 37°C for 1 hr. Colorimetric change was measured using a microtiter plate reader (Spectra Max M5; Molecular Devices, Sunnyvale, CA, USA). The biofilm formation ratio was expressed as a percentage of the untreated control.

6. Statistical analysis
The data were analyzed using a Student’s t-test. All statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). All data were considered significant at p<0.05.

Results

1. Inhibitory effects of resveratrol on biofilm formation of C. albicans

Figure 2 shows the inhibitory effects of resveratrol on biofilm formation by C. albicans strain SC5314. Resveratrol minimally inhibited growth of biofilm when it was added to the medium at the adherent stage (Fig. 2a), demonstrating a statistically significant inhibitory effect only at 50 or 100 μg/ml, however, compared to in the control group: 50 μg/ml of resveratrol was the most effective in inhibiting growth of C. albicans biofilm at this stage.

Upon treating the biofilm with resveratrol at the developmental stage, C. albicans growth was inhibited in a dose-dependent manner (Fig. 2b), with significant inhibition observed at a concentration of 25–200 μg/ml. Growth was considerably reduced by approximately 20% in the presence of 200 μg/ml resveratrol compared to in its absence.

Prevention of biofilm formation by resveratrol at the sustained stage of growth was
observed at 50–200 μg/ml (Fig. 2c). Although 6.25 μg/ml resveratrol also inhibited growth of biofilm, the extent of inhibition was less than that with 50–200 μg/ml resveratrol. Biofilm growth by *C. albicans* at this stage was distinctly reduced by >30% in the presence of 200 μg/ml resveratrol in comparison with in its absence. Thus, resveratrol inhibited biofilm formation most effectively when it was added at the developmental or sustained stages of growth.

2. Effects of resveratrol on disruption of matured biofilm

Next, the effect of resveratrol on disruption of matured biofilm was determined. Cell viability of *C. albicans* biofilm was significantly reduced in the presence of 6.25–100 μg/ml resveratrol (p<0.05). Resveratrol at 50 μg/ml exhibited distinctly greater inhibition (>30%) than in its absence (Fig. 3). No significant difference was observed between the control and biofilm treated with 200 μg/ml resveratrol.

![Fig. 2](image1.png)

**Fig. 2** Effects of resveratrol on three stages of *C. albicans* biofilm development. *Candida albicans* yeast (1×10^7 cells/ml) was incubated with various concentrations of resveratrol at 37°C for each of three stages of biofilm formation. Results are expressed as percentage of untreated control. Data represent mean±S.D. of three independent experiments performed in quadruplicate. Student’s *t*-test *p*<0.05, **p**<0.01, ***p***<0.001 vs. resveratrol untreated control, respectively. (a) Effects of resveratrol at adherent stage of *C. albicans* biofilm development. (b) Effects of resveratrol at developmental stage of *C. albicans* biofilm development. (c) Effects of resveratrol at sustained stage of *C. albicans* biofilm development.

![Fig. 3](image2.png)

**Fig. 3** Effects of resveratrol on established mature biofilm of *C. albicans*. 24-hr-old *C. albicans* biofilm was exposed to various concentrations of resveratrol at 37°C for 24 hr in 96-well plates. Results are expressed as percentage of untreated control. Data represent mean±S.D. of three independent experiments performed in quadruplicate. Student’s *t*-test *p*<0.05, **p**<0.01 vs. resveratrol untreated control, respectively.
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Discussion

*Candida albicans* is the principal species of fungi isolated from patients with oropharyngeal candidiasis, which is a frequent symptom of human immunodeficiency virus infection. Few classes of antifungal drugs are currently applicable in the clinical treatment of oral or systemic candidiasis. Moreover, overuse of such medicines may lead to drug-resistant mycosis resulting from the emergence of drug-resistant *Candida* strains. The evaluation of novel antifungal agents as alternative drug therapies is required due to the toxic side-effects of antifungal drugs and the emergence of drug-resistant *Candida* species\(^{13}\). The present study showed that *C. albicans* biofilm formation was reduced in the presence of resveratrol. Additionally, resveratrol was effective in the control of established biofilms of *C. albicans*. Dose-dependent growth inhibition of *C. albicans* by resveratrol was observed when the biofilm was treated at the developmental stage, suggesting that resveratrol reduces formation of the *C. albicans* basic yeast cell poly-layer, filamentation, and hyphal elongation. However, less attenuation was observed when it was added at the adherent step of biofilm formation, indicating that a high concentration of resveratrol potentially intervenes at the intermediate phase of biofilm formation\(^5\). Although there was no significant difference between the control and biofilm treated with 200 µg/ml resveratrol in the biofilm disruption assay, partial aggregation of the biofilm was observed (data not shown), suggesting that a high concentration of resveratrol affects cell surface specificity by influencing proteins essential for *C. albicans* growth\(^{21}\).

*Candida albicans* biofilm is composed of a mixed structure of yeast cells and hyphal elements; therefore, biofilm development is also likely to play an important role in the dimorphic switch. Two major signaling pathways, the Cph1-mediated mitogen-activated protein kinase and cAMP-dependent protein kinase A pathways, are well-characterized signaling transduction pathways that control dimorphic regulation\(^{12}\). Resveratrol may affect signal transduction, and additional study is needed to clarify the molecular mechanisms by which resveratrol inhibits *C. albicans* biofilm formation.

Conclusion

Resveratrol has the potential to serve as an anti-*Candida* treatment and preventive tool. It inhibits existing or forming *C. albicans* biofilms, suggesting that it offers a promising candidate for the development of new antifungal treatments.

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References

1) Baillie GS, Douglas LJ (1999) Role of dimorphism in the development of *Candida albicans* biofilms. J Med Microbiol 48:671–679.
2) Baur JA, Sinclair DA (2006) Therapeutic potential of resveratrol: the in vivo evidence. Nat Rev Drug Discov 5:493–506.
3) Berridge MV, Herst PM, Tan AS (2005) Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev 11:127–152.
4) Blankenship JR, Mitchell AP (2006) How to build a biofilm: a fungal perspective. Curr Opin Microbiol 9:588–594.
5) Cavalheiro M, Teixeira MC (2018) *Candida* biofilms: threats, challenges, and promising strategies. Front Med (Lausanne) 5:28.
6) Correa MG, Pires PR, Ribeiro FV, Pimentel SP, Cirano FR, Napimoga MH, Casati MZ, Casarin RCV (2018) Systemic treatment with resveratrol reduces the progression of experimental periodontitis and arthritis in rats. PLoS One 13:e0204414.
7) de Repentigny L, Lewandowski D, Jolicoeur P (2004) Immunopathogenesis of orophary-
geal candidiasis in human immunodeficiency virus infection. Clin Microbiol Rev 17: 729–759.
8) Gulati M, Lohse MB, Ennis CL, Gonzalez RE, Perry AM, Bapati P, Arevalo AV, Rodriguez DL, Nobile CJ (2018) In vitro culturing and screening of Candida albicans biofilms. Curr Protoc Microbiol 50:e60.
9) Hogan DA, Sundstrom P (2009) The Ras/cAMP/PKA signaling pathway and virulence in Candida albicans. Future Microbiol 4: 1263–1270.
10) Hsieh YH, Zhang JH, Chuang WC, Yu KH, Huang XB, Lee YC, Lee CI (2018) An in vitro study on the effect of combined treatment with photodynamic and chemical therapies on Candida albicans. Int J Mol Sci 19:337.
11) Ignatowicz E, Baer-Dubowska W (2001) Resveratrol, a natural chemopreventive agent against degenerative diseases. Pol J Pharmacol 53:557–569.
12) Inglis DO, Sherlock G (2013) Ras signaling gets fine-tuned: regulation of multiple pathogenic traits of Candida albicans. Eukaryot Cell 12:1316–1325.
13) Kanafani ZA, Perfect JR (2008) Antimicrobial resistance: resistance to antifungal agents: mechanisms and clinical impact. Clin Infect Dis 46:120–128.
14) Lo HJ, Kohler JR, DiDomenico B, Loebenberg D, Cacciapuoti A, Fink GR (1997) Nonfilamentous C. albicans mutants are avirulent. Cell 90:939–949.
15) Lohse MB, Gulati M, Johnson AD, Nobile CJ (2018) Development and regulation of single- and multi-species Candida albicans biofilms. Nat Rev Microbiol 16:19–31.
16) Lohse MB, Gulati M, Valle Arevalo A, Fishburn A, Johnson AD, Nobile CJ (2017) Assessment and optimizations of Candida albicans in vitro biofilm assays. Antimicrob Agents Chemother 61:e02749-16.
17) Nobile CJ, Johnson AD (2015) Candida albicans biofilms and human disease. Annu Rev Microbiol 69:71–92.
18) Odds FC (1987) Candida infections: an overview. Crit Rev Microbiol 15:1–5.
19) Okamoto-Shibayama K, Sato Y, Azuma T (2010) Resveratrol impaired the morphological transition of Candida albicans under various hyphae-inducing conditions. J Microbiol Biotechnol 20:942–945.
20) Repossi G, Das UN, Eynard AR (2020) Molecular basis of the beneficial actions of resveratrol. Arch Med Res 51:105–114.
21) Ruiz-Herrera J, Elorza MV, Valentín E, Sentandreu R (2006) Molecular organization of the cell wall of Candida albicans and its relation to pathogenicity. FEMS Yeast Res 6:14–29.
22) Sudbery P, Gow N, Berman J (2004) The distinct morphogenic states of Candida albicans. Trends Microbiol 12:317–324.
23) Tumbarello M, Posteraro B, Trecarichi EM, Fiori B, Rossi M, Porta R, de Gaetano Donati K, La Sorda M, Spanu T, Fadda G, Cauda R, Sanguinetti M (2007) Biofilm production by Candida species and inadequate antifungal therapy as predictors of mortality for patients with candidemia. J Clin Microbiol 45:1843–1850.
24) Uppuluri P, Pierce CG, Thomas DP, Bubeck SS, Saville SP, Lopez-Ribot JL (2010) The transcriptional regulator Nrg1p controls Candida albicans biofilm formation and dispersion. Eukaryot Cell 9:1531–1537.

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