Antifungal Activity of Dehydrocurvularin for *Candida* spp. through the Inhibition of Adhesion to Human Adenocarcinoma Cells

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ABSTRACT

Cell adhesion plays a crucial role in candidiasis through invasion of the human body and obtaining resistance to drugs by forming biofilms. Cell adhesion thus is a critical target for combating candidiasis by preventing the entry of fungal hyphae into the epithelium. We report here that dehydrocurvularin (**1**), isolated from the marine-derived fungus *Curvularia aeria*, exhibited anti-fungal activities for *Candida albicans* and *Candida auris*. This compound also prevented the adherence of *C. albicans* to human adenocarcinoma cells. Real-time RT-PCR analysis showed that exposure to **1** results in decreased expression of *HWP1, EFG1*, and *ECE1*, genes involved in *Candida* adhesion to epithelial cells and hyphal morphogenesis.

Candidiasis is caused by fungi of the genus *Candida*, which are common commensal organisms in the human body. In immunocompromised hosts, *Candida* spp. can infect by invading and damaging epithelial cells; this entry often occurs via medical devices (ref. 1). Classically, candidiasis was caused primarily by *Candida albicans*, but *Candida auris*, newly discovered in 2009 in the external aural canal of a patient in Japan. (ref. 2), increasingly has been identified in cases of candidiasis. Though azole- and polyene-type antifungal agents are used clinically to treat candidiasis, side effects and increasing resistance to these compounds are ongoing concerns. Antifungal drugs with mechanisms of action distinct from those of azole- or polyene-type compounds are needed.

The toxicity of candidiasis is exhibited through the process of adhesion to host cells, fungal proliferation, and the formation of mature biofilms. *C. albicans* is a dimorphic fungus that transforms from a yeast to a hyphal form; resistance to drugs may be obtained by maturing biofilms. Therefore, cell adhesion is a potential target for novel antifungal agents.

The endocannabinoid anandamide shows antifungal activity against *C. albicans.* Treatment of *C. albicans* with anandamide strongly reduces the adherence of this fungus to cervical epithelial cells, making anandamide and its derivatives possible drugs for the co-treatment of infections caused by this organism. On the other hand, anandamide does not appear to inhibit biofilm formation on polystyrene plastic surfaces. This observation suggests that anandamide shows antifungal activity with cell surface specificity (ref. 3).

Natural products of microbial origin are attractive candidates for novel antifungal agents. However, only a few compounds with anti-candidiasis activity targeting adhesion have been discovered. Screens for natural compounds focused on cell adhesion activity are expected to identify novel antifungal leads. In the present study, we report the antifungal properties of

dehydrocurvularin (**1**). The minimum inhibitory concentration (MIC) values and anti-adhesion activity of **1** for *C. albicans* were investigated. Additionally, real-time reverse transcription-polymerase chain reaction (RT-PCR) analysis was used to assess the effect of **1** on the expression of genes related to *Candida* adhesion to epithelial cells and hyphal morphogenesis.

Chemical investigation was performed for marine-derived fungi that have been deposited in our laboratory. The crude $CHCl_3$ extract of an isolate of *Curvularia aeria* was selected from the screening about antifungal activity for *C. albicans* and *C. auris*. The minimal inhibitory concentrations (MICs) of *C. aeria* $CHCl_3$ extract against *C. albicans* and *C. auris* were both 100 μg/mL. This $CHCl_3$ extract was subjected to fractionation, resulting in the isolation of two aromatic polyketides, dehydrocurvularin (**1**) and curvularin (**2**) (Figure. 1). The structures of these compounds were defined on the basis of spectroscopic data, as well as by comparison with published data (ref. 4). These molecules consist of 12-membered-ring ketolactones with 1,3-dihydroxybenzene and previously were assessed for MICs against *Saccharomyces cerevisiae* (ref. 5).

The MIC values of **1** and **2** against *C. albicans* and *C. auris* were determined in 96-well plates according to methods described in the CLSI Document M27-A3, with minor modifications (ref. 6). The MIC values of compound **1** were MIC of 250 μg/mL against both *Candida* species. On the other hand, **2** did not show inhibitory activity against either *Candida* species. *C. auris* is inherently resistant to azole-type antifungal drugs, such that the MIC of fluconazole for *C. auris* is over twenty times than that for *C. albicans* (3.12 and 0.125 μg/mL, respectively). The MIC value for *C. albicans* and *C. auris* of **1** suggested that **1** affects *Candida* spp. regardless of azole resistance.

Since **1** showed antifungal activity, we next investigated the compound's anti-adhesion activity in *C. albicans*. Adhesion is the first step in initiation of infection by the fungus. *C. albicans*-related

77 bloodstream infections often are associated with infection of central venous catheters, which is
78 facilitated by microbial adhesion and biofilm formation (ref. 7). Therefore, anti-adherence activity
79 on 24-well microplates was tested. *C. albicans* was cultured (in 24-well flat-bottom microplates
80 for 3 h at 37 °C), in the absence of cultured cells, in cell culture medium with or without **1** (Figure
81 2a).

82 Cell growth and hyphal formation was observed in the control cultures (i.e., lacking **1**). In the
83 presence of 250 μg/mL of **1**, *C. albicans* cell growth and hyphal formation were inhibited. This
84 result was consistent with the results obtained for the MIC analysis of **1**.

85 Next, we investigated the effect of **1** on the adhesion of *C. albicans* to the surface of A549 human
86 lung adenocarcinoma cells; the co-culture of the fungus and cultured cells imitated infection of
87 human lung cells. A549 cells were grown to confluence in 24-well flat-bottom plates. *C. albicans*
88 suspension in the presence of 62.5 μg/mL of **1** was applied on A549 cells and the plates then were
89 incubated for another 7 h at 37 °C. This concentration of **1** was chosen because this level did not
90 show any adverse effects on *C. albicans* proliferation activity, as determined in the MIC test. To
91 observe *C. albicans* clearly, fungi were stained with Calcofluor white and visualized by
92 fluorescence microscopy. The results are shown in Figures 2b and 2c. Compound **1** at 62.5 and
93 125 μg/mL reduced the number of *C. albicans* adhering to A549 cell to 55.7% and 30.5%. Notably,
94 these concentrations of **1** were lower than the MIC.

95 To understand the anti-adhesive effects of **1** on *C. albicans*, we assessed gene expression in fungi
96 exposed for 7 h to **1** at 62.5 μg/mL, the concentration that showed anti-adhesion activity against
97 *C. albicans*. Gene expression analysis was performed by real-time RT-PCR for genes relevant to
98 biofilm formation, adherence, and hyphal morphogenesis. Specifically, the mRNA levels of *EFG1,*
99 *TEC1, BCR1, HWP1*, and *ECE1* were examined (Table S1). The products of *EFG1, TEC1*, and

BCR1 participate in cyclic adenosine monophosphate (cAMP) signalling, a system that contributes to the yeast-to-hypha transition (ref. 3, 8–10). The products of *HWP1* and *ECE1* regulate *C. albicans* cell adhesion: *HWP1* encodes a cell wall mannose protein essential for normal growth of the mycelium; *ECE1* encodes candidalysin, a peptide toxin that activates epithelial cells (ref. 3) The results (Figure 3) showed that the expression of *EFG1, TEC1, ECE1*, and *HWP1* are significantly decreased in the presence of 62.5 μg/mL **1** (compared to cultures grown in the absence of **1**). Among these genes, *BCR1* expression levels were unchanged. Gene expressions of *NRG1, TUP1, UME6* and *RAS1* were also investigated. *NRG1, TUP1, UME6* encodes filamentous growth of *C. albicans* (ref. 11). Ras1 is an upstream regulator of cAMP/Efg1 signal transduction pathway. The expression levels of these genes were unchanged. These results suggested that **1** showed antifungal activity *via* an inhibition of adhesion.

Anti-adhesive activity of **1** may be mediated by effects on more than one pathway. The downregulation of *EFG1* and *TEC1* is consistent with the inhibition of the yeast-to-hypha transition. Anti-cell adhesion activity also is expected, given the observed downregulation of *HWP1* and *ECE1*. Comparison of the effects of **1** and anandamide's effects on the adhesion of filamentous *C. albicans* to cells revealed that *HWP1* and *ECE1*, two adhesion-related genes, were downregulated in common between the two compounds.

In conclusion, we report that dehydrocurvularin (**1**) exhibits antifungal activity against both *C. albicans* and *C. auris*. Hyphal growth and cell adhesion were decreased in *C. albicans* grown in the presence of **1** at concentrations exceeding 62.5 μg/mL. This inhibition was achieved by decreased levels of transcripts encoding proteins involved in cell–cell interaction and those known to regulate hyphal morphogenesis. Compound **1** also showed antifungal activity against the non-dimorphic *Candida* species *C. auris*. Therefore, antifungal activities mediated by changes in cell

adhesion are expected to be of value in the development of new drugs with clinical activity against candidiasis.

Supplementary information

Supplementary information is available at Journal of Antibiotics website https://

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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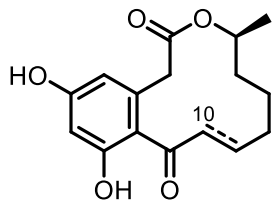

1: dehydrocurvularin Δ^{10}
2: curvularin

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163 **Fig. 1** Structures of dehydrocurvularin (**1**) and curvularin (**2**).

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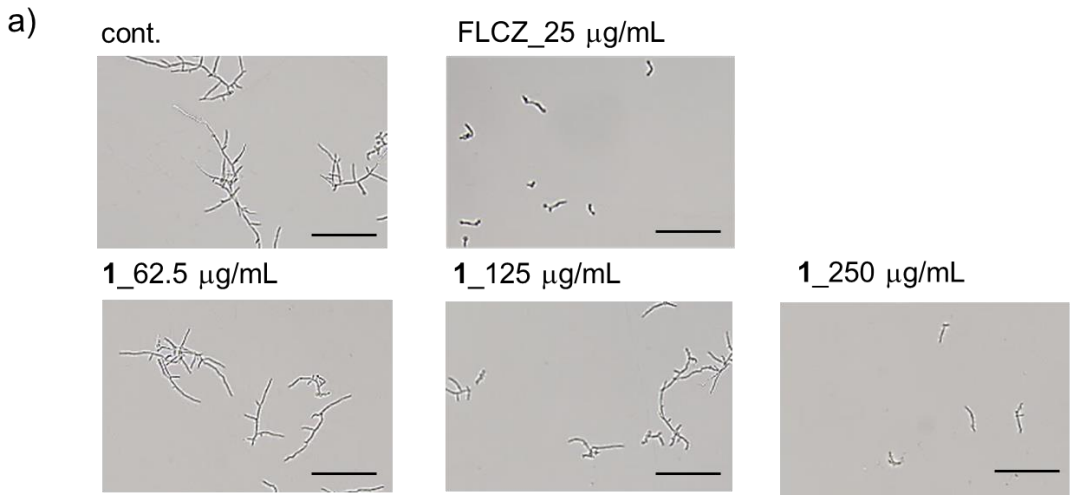
a)
cont.
FLCZ_25 μg/mL
1_62.5 μg/mL
1_125 μg/mL
1_250 μg/mL

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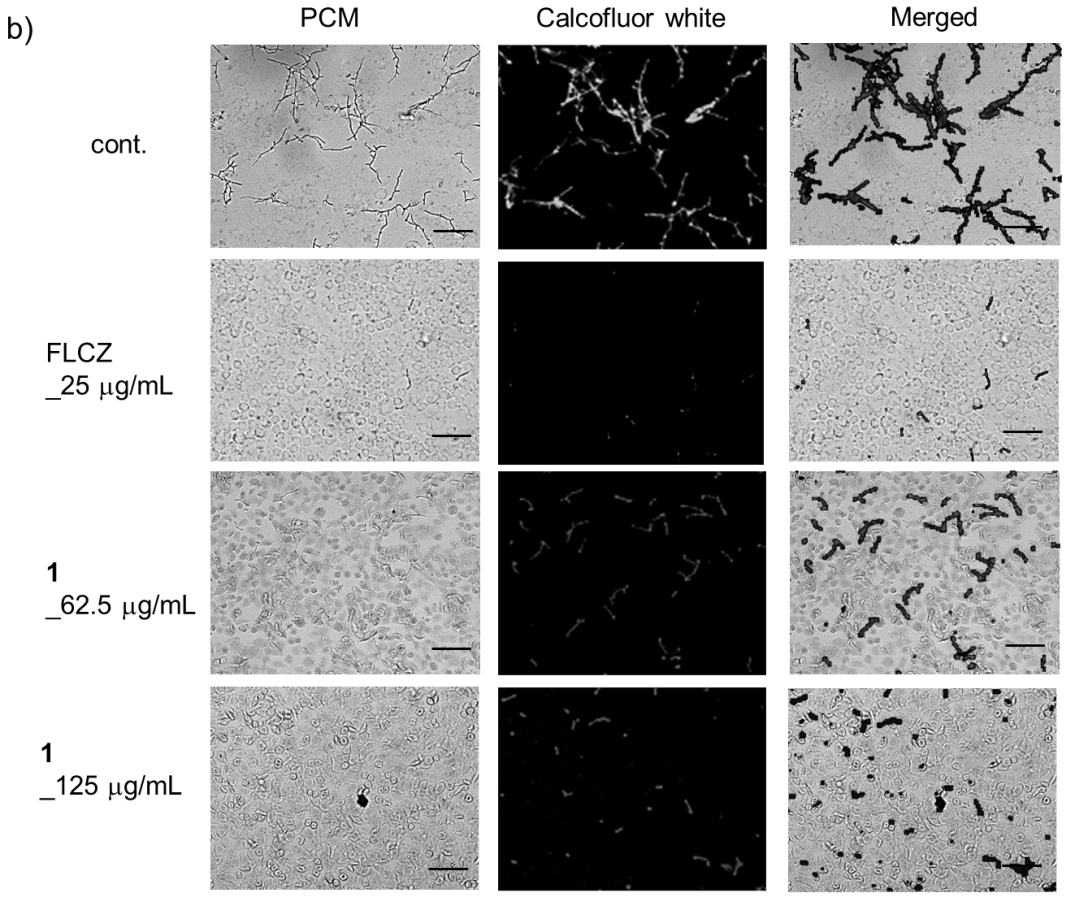
b)
PCM
Calcofluor white
Merged
cont.
FLCZ
_25 μg/mL
1
_62.5 μg/mL
1
_125 μg/mL

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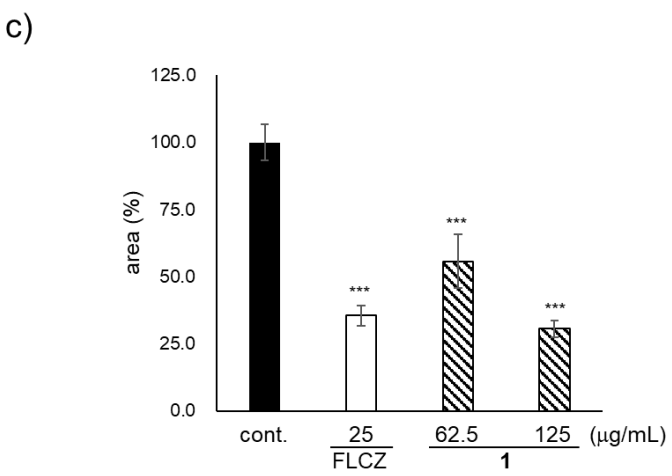
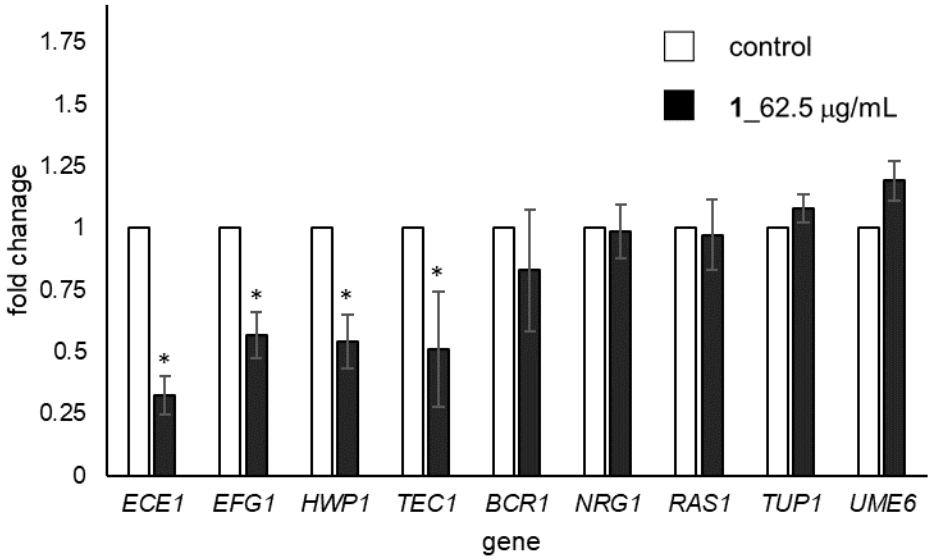


Fig. 2 Anti-adherence activity of **1**. (a) Anti-adherence activity for *C. albicans* on plastic plates. *C. albicans* were grown in RPMI 1640 medium at a range of concentrations of **1**. At the indicated time (7 h), the images were obtained using a light microscope (FLCZ: fluconazole, scale bars: 100 μm). (b) Effect of **1** on the adhesion of *C. albicans* to A549 cells. The A549 cells were co-incubated with *C. albicans* for 7 h. Adherent *C. albicans* cells were stained with Calcofluor white (PCM: Phase Contrast microscopy, scale bars: 100 μm). (c) The number of adherent cells was determined by counting the stained area in the pictures. Bars represent means ± SD ***$P < 0.001$ (n = 3).



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178 **Fig. 3** Modulation of expression of hypha-specific genes in *C. albicans* in response to treatment
179 with **1**. *C. albicans* was treated with 62.5 μg/mL of **1** for 7 h at 37 °C. Transcriptional levels of
180 genes are indicated as a fold change relative to that in the control. Bars represent means ± SD **P*
181 < 0.05 (n = 4).

182

Figure legends

Fig. 1 Structures of dehydrocurvularin (**1**) and curvularin (**2**).

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