Blocking of Candida albicans biofilm formation by cis-2-dodecenoic acid and trans-2-dodecenoic acid

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Candida is an important opportunistic human fungal pathogen. Infections caused by Candida albicans are related to the formation of a biofilm. The biofilm enhances the resistance of the C. albicans defence system, increases its resistance to antifungal drugs and induces increased drug tolerance, making clinical care more challenging. The in vitro activity of cis-2-dodecenoic acid (BDSF; a diffusible signal factor from Burkholderia cenocepacia) and trans-2-dodecenoic acid (trans-BDSF) against C. albicans growth, germ-tube germination and biofilm formation was estimated by absorbance measurements and microscopic assessments. C. albicans biofilms were prepared using a static microtitre plate model. Quantitative analysis of biofilm formation was performed using a 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide reduction assay to evaluate the effect of different concentrations of BDSF and trans-BDSF at different stages of biofilm formation. Reductions in biofilm structure and formation were visualized by inverted microscopy. Real-time RT-PCR was employed to estimate the mRNA expression levels of the hyphae-specific genes HWP1 and ALS3. It was found that 30 μM of either BDSF or trans-BDSF reduced germ-tube formation by approximately 70 % without inhibiting yeast growth. Yeast growth was strongly repressed by the exogenous addition of 300 μM BDSF and trans-BDSF at 0 and 1 h after cell attachment, with biofilm formation being reduced by approximately 90 and 60 %, respectively. BDSF and trans-BDSF were more effective against biofilm formation than farnesol and the diffusible signal factor cis-11-methyl-2-dodecenoic acid. None of the four drugs was able to destroy pre-formed biofilms. Real-time RT-PCR analysis showed that HWP1 was downregulated by approximately 90 % and ALS3 was downregulated by 70–80 % by 60 μM BDSF and trans-BDSF, implying that BDSF and trans-BDSF block C. albicans biofilm formation by interfering with the morphological switch. These results suggest that BDSF and trans-BDSF are potentially useful therapeutic agents worthy of further study.

INTRODUCTION

Members of the genus Candida are fungi that are frequently pathogenic and are capable of causing different kinds of infections (Cao et al., 2005). Candida albicans is the most common species associated with candidiasis, accounting for 76 % of all cases (Pierce et al., 2009). C. albicans forms biofilms, which are composed of a mixture of cell types, including yeast, pseudohyphal and hyphal cells (Borecká-Melkusová et al., 2009; Douglas, 2003; Kumamoto, 2002).

C. albicans is polymorphic, with two distinct morphological forms: yeast and filamentous. The ability of C. albicans to cause disease has been linked to dimorphic switching (Lo et al., 1997), a process that is induced by many different environmental factors (Brown & Gow, 1999; Brown et al., 1999) and by antifungal agents. Farnesol, produced by planktonic cultures of C. albicans, blocks the yeast-to-hyphal conversion required for biofilm formation (Molero et al., 1998). Ramage et al. (2002a) found that the effect of farnesol on biofilm development was time-dependent and that, once hyphal formation had been initiated, biofilm growth was not inhibited.

The diffusible signal factor cis-11-methyl-2-dodecenoic acid (DSF) can bind to the farnesol receptor of C. albicans, leading to an arrest in filamentation (Wang et al., 2004).
Low-molecular-mass fatty acids, including the diffusible signal factor cis-2-dodecenoic acid from *Burkholderia cepacia* (BDSF), have been shown to regulate the *C. albicans* dimorphic switch (Boon *et al.*, 2008; Wang *et al.*, 2004). Further analyses have shown that *C. albicans* germ-tube formation is strongly inhibited by exogenous addition of physiological levels of BDSF (Boon *et al.*, 2008). The structures of BDSF and DSF are similar, with both molecules having a cis double bond at position 2 of the 11-carbon chain. The fatty acid messenger cis-2-decanoic acid produced by *Pseudomonas aeruginosa* has been reported to be responsible for inducing dispersion in *C. albicans* biofilms at concentrations ranging from 1.0 to 10.0 mM, whilst trans-2-decanoic acid was active only at millimolar concentrations (Davies & Marques, 2009). In the present study, we investigated the potential inhibitory activity of BDSF and trans-BDSF in *C. albicans* biofilm formation.

**METHODS**

**Chemicals.** Farnesol, 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT) and menadione used for biofilm quantification were purchased from Sigma. DSF, BDSF and trans-BDSF were synthesized as described previously (Wang, 2006). Stocks of 0.3 M DSF, farnesol, BDSF and trans-BDSF were prepared individually in a mixture of equal volumes of methanol and sterile H2O and stored at −20°C until used. Subsequent dilutions of BDSF and trans-BDSF were made in RPMI 1640 (Gibco) supplemented with 1-glutamine and buffered with 165 mM MOPS (Bio Basic) or yeast peptone dextrose (YPD) liquid medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose]. XTT was prepared at 0.5 mg ml−1 in sterile PBS. The solution was then filter-sterilized through a 0.22 μm pore-size filter. Prior to each assay, menadione (10 mM prepared in acetone) was added to the XTT to a final concentration of 1 μM.

**C. albicans strain and culture conditions.** The standard strain *C. albicans* SC5314 cultivated on Sabouraud Dextrose Agar medium (SDA: 1 % peptone, 4 % dextrose, 2 % agar powder, pH 7.0) was used. *C. albicans* SC5314 cultivated on Sabouraud Dextrose Agar medium solution was then filter-sterilized through a 0.22 μm pore-size filter and buffered with 165 mM MOPS (Bio Basic) or yeast peptone dextrose (YPD) liquid medium [1 % (w/v) yeast extract, 2 % (w/v) peptone, 2 % (w/v) dextrose]. XTT was prepared at 0.5 mg ml−1 in sterile PBS. The solution was then filter-sterilized through a 0.22 μm pore-size filter. Prior to each assay, menadione (10 mM prepared in acetone) was added to the XTT to a final concentration of 1 μM.

**Microscopic assessment of *C. albicans* germ-tube formation.** To test the effect of various signal molecules on *C. albicans* germ-tube formation, *C. albicans* SC5314 (1 × 105 cells ml−1 in RPMI 1640 with MOPS) was incubated with or without BDSF or trans-BDSF at 37°C without shaking. Hyphae induction for microscopic quantification was performed in six-well, flat-bottomed, non-treated plastic tissue-culture plates (Haimen). After a 3 h incubation period, germ-tube formation in four small areas per plate (each containing 100 *C. albicans*) was counted using an inverted phase-contrast fluorescence microscope.

**C. albicans yeast growth.** The stock solutions of farnesol, BDSF, trans-BDSF and DSF were diluted using YPD medium to the required concentrations (6, 60, 120 and 600 μM, respectively). A loopful of *C. albicans* cells from an SDA plate was suspended in YPD medium and a 1.2 ml aliquot of the suspension containing approximately 2 × 10⁴ cells ml−1 and 1.2 ml YPD medium supplemented with different drugs were added to 50 ml centrifuge tubes. YPD medium with no added drugs was used as a negative control. After incubation at 37°C with shaking at 160 r.p.m. for 24 h, the OD₆₀₀ was recorded.

**C. albicans biofilm formation.** Biofilms were formed and quantified on commercially available pre-sterilized, polystyrene, flat-bottomed, 96-well microtitre plates (Costar 3599; Corning). The 0.3 M stock solutions of farnesol, BDSF, trans-BDSF and DSF were diluted using RPMI 1640 in tenfold dilutions to give final concentrations of the drugs ranging from 0.003 to 0.3 μM. Aliquots of 100 μl of the suspensions containing approximately 2 × 10⁶ cells ml−1 in RPMI 1640 were seeded into selected wells of the microtitre plate and incubated for 1, 2 and 4 h at 37°C. After the initial incubation, the medium was aspirated, and unattached cells were removed by washing the wells three times with sterile PBS. The drugs (100 μl) were then added to different wells of the adherent cells to give final concentrations of 3, 30 and 300 μM. A row with 100 μl RPMI 1640 without any drugs was included to serve as a positive control for biofilm growth. Farnesol, BDSF, trans-BDSF and DSF were also added individually to different standardized suspensions before being added to the microtitre plate (time 0). The microtitre plate was covered with its lid, sealed with Parafilm and incubated at 37°C for another 24 h.

To examine the inhibitory effect on pre-formed biofilms, *C. albicans* biofilms were formed for 24 h at 37°C and the wells were then washed twice with sterile PBS. Aliquots of 100 μl of RPMI 1640, each containing the different drugs in serially tenfold-diluted concentrations from 300 to 3 μM, were added to the wells. To serve as a positive control for biofilm growth, a row of wells containing 100 μl RPMI 1640 without any drugs was included. The plate was incubated at 37°C for another 24 h. To visualize the *in vitro* biofilm, cells were removed from the surrounding medium. Biofilm formation was measured at each time interval using an XTT-reduction assay and concurrently assessed by fluorescence microscopy at × 400 magnification.

**XTT-reduction assay.** After incubating the cells for the required period, the medium was thoroughly removed and each well was washed three times with 200 μl sterile PBS. A colorimetric XTT-reduction assay was used for the semi-quantification of fungal-cell viability in the wells (Kuhn *et al.*, 2003). To each pre-washed well on the microtitre plate, 80 μl XTT/menadione was added and the plate was incubated in the dark at 37°C for 2 h. Metabolically active cells can reduce XTT, yielding a water-soluble, coloured formazan product (da Silva *et al.*, 2008). After incubation, 75 μl XTT/menadione solution was transferred to a new microtitre plate and the colorimetric change (ΔA₆₃₀) was read.

**Real-time RT-PCR.** Primers for the hyphal-specific genes *HWPI* and *ALS3* genes, as well as the reference gene *ACT1*, are shown in Table 1. Fresh SC5314 strains in YPD with 100 μg chloramphenicol ml⁻¹ were collected and diluted to a density of approximately 2 × 10⁶ c.f.u. ml⁻¹ in RPMI 1640, and 1 ml aliquots of the suspension were added to each of the wells in a sterile six-well plate (Costar 3516; Corning). After incubation at 37°C without shaking for 1 h, 1 ml RPMI 1640 containing 60 or 120 μM BDSF or trans-BDSF was added to the corresponding well. A culture without any added drug was used as a positive control. The plate was incubated at 37°C without shaking for a further 24 h. Biofilm supernatants were removed and the biofilm was washed gently with sterile PBS. Biofilm cells were removed from the bottom with a sterile scraper and biofilm RNA was obtained using an EASYspin Yeast RNA Fast Extraction kit (Beijing Yuanpinghao Biotechnology Co.) and treated with DNase (Tiangen Biotech Co.), following the manufacturer’s protocol.
cDNA was synthesized using a T7erVart First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer’s protocol. Following synthesis, the cDNA was diluted 1:16 with sterile MilliQ water (Millipore) and used as a real-time RT-PCR template. A SYBR Premix Ex Taq kit (TaKaRa) was used with a Stratagene MX3000P System for real-time RT-PCR. A dilution series of cDNA stock solutions from 1:4 to 1:1024 was used to construct a standard curve for each primer pair. The reaction procedure comprised incubation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s, 55 °C for 20 s and 72 °C for 30 s. After this process was complete, melting curves were obtained for 95 °C for 15 s, 55 °C for 30 s and 95 °C for 30 s. Each primer pair showed a uniform dissociation curve, confirming that the primer pairs worked well. The housekeeping actin gene ACT1 was used to normalize the real-time RT-PCR data and to calculate the relative fold changes in gene expression. Independent reactions were repeated four times with the extraction of different RNA samples. Means ± SD were determined using GraphPad Prism software.

**Statistical analysis.** All experiments were performed in triplicate and the results were expressed as means ± SD. For XTT readings, the mean A490 values were calculated from multiple replicate wells for a given condition; background was eliminated by subtracting the A490 value of blank wells. Statistical analyses of the differences between mean values obtained for the experimental groups were performed using Student’s t-test. P < 0.05 was considered significant.

**RESULTS**

**BDSF and trans-BDSF inhibit germ-tube formation in *C. albicans***

BDSF is structurally related to farnesol and its molecular structure is very similar to that of DSF. Both farnesol and DSF have been shown to inhibit germ-tube formation in *C. albicans* (Hogan *et al.*, 2004; Wang *et al.*, 2004). Boon *et al.* (2008) found that BDSF was a highly potent inhibitor of *C. albicans* hyphal growth. In this study, to determine the potential inhibitory activity of *trans*-BDSF in *C. albicans*, *trans*-BDSF was added to fresh fungal yeast cells and compared quantitatively with the activity of DSF, BDSF and farnesol using medium and growth conditions described previously (Hogan *et al.*, 2004); methanol was used as the solvent control. After incubation at 37 °C for 3 h, >80% of the *C. albicans* cells in the control well formed germ tubes. Farnesol at a concentration of 3 μM reduced the length of the germ tubes slightly, but had no apparent effect on germination rate. In contrast, 3 μM BDSF and *trans*-BDSF significantly reduced germ-tube germination by approximately 60% (Fig. 1a), in agreement with a previous report for BDSF (Boon *et al.*, 2008). Even at 0.3 μM concentrations, BDSF and *trans*-BDSF reduced germ-tube formation by approximately 10%, outperforming the close structural DSF analogue (results not shown). At final concentrations of 30 μM, none of the drugs inhibited yeast growth significantly (Fig. 1b); however, BDSF and *trans*-BDSF reduced germ-tube formation significantly by approximately 70% at this concentration, whilst farnesol and DSF had no obvious effect. The data suggested that, even at low concentrations, both BDSF and *trans*-BDSF are highly potent inhibitors of *C. albicans*.

**Table 1. PCR primers for real-time RT-PCR**

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<th>Gene</th>
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<th>Sequence (5’→3’)</th>
<th>Sequence coordinates (nt)</th>
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<td></td>
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**Fig. 1.** Comparison of the effect of BDSF and *trans*-BDSF on *C. albicans* germ-tube formation and yeast growth. (a) Inhibitory activity of BDSF, *trans*-BDSF, farnesol and DSF on germ-tube formation in *C. albicans* 3 h after induction. The experiment was performed twice and at least 400 cells were counted for each treatment. (b) Effect of the four drugs on *C. albicans* yeast-cell growth. The OD<sub>600</sub> was determined after 24 h growth at 37 °C with agitation. Controls had no drug added.
germ-tube germination. BDSF and trans-BDSF were the most effective inhibitors, followed by DSF and farnesol, in agreement with previous observations (Hogan et al., 2004). At 300 μM, farnesol reduced germ-tube formation by approximately 60%, whilst BDSF and trans-BDSF inhibited formation by approximately 90%.

**Effects of BDSF and trans-BDSF on C. albicans yeast growth**

Prior studies showed that farnesol did not alter the growth of C. albicans yeast (Henriques et al., 2007), and we found that farnesol and DSF showed a similar trend. At low concentrations (3, 30 and 60 μM), the four chemicals tested had no apparent effect on yeast growth. Even at 300 μM, farnesol and DSF had no obvious effect on yeast-cell growth; however, at this concentration, both BDSF and trans-BDSF inhibited yeast-cell growth significantly by approximately 90% (Fig. 1b).

**BDSF and trans-BDSF have an inhibitory effect on C. albicans biofilm formation**

BDSF and trans-BDSF were used to treat adherent cell populations at 0, 1, 2, 4 and 24 h to determine whether different concentrations (3, 30 and 300 μM) could adversely affect C. albicans biofilm formation. Semi-quantitative measures of biofilm formation were made using an XTT-reduction assay. To complement the XTT-reduction assay results, microscopy was also used. The microscopic analysis of the control biofilm and of those treated with the four different inhibitors is shown in Fig. 2. The four drugs displayed various effects at each of the tested concentrations (3, 30 and 300 μM) over different time periods (0, 1, 2, 4 and 24 h) during C. albicans biofilm formation. The positive controls (biofilms formed in the absence of any drugs) for C. albicans SC5314 had the typical biofilm architecture composed of intertwining mycelium (Fig. 2).

The addition of 3 μM farnesol or DSF at the time of cell attachment (0 h) had no obvious inhibitory effect; however, 30 μM slightly reduced and 300 μM strongly reduced the quantity of biofilm formed (Fig. 2). In contrast to this result, when added at 0 h, 3 μM BDSF and trans-BDSF significantly decreased the density of biofilms, which were composed of thin mycelium; at 30 μM, BDSF and trans-BDSF noticeably inhibited biofilm formation (P<0.05), causing scantly biofilms, and at 300 μM they arrested yeast C. albicans growth completely (Fig. 2). These observations were reflected in the A490 values obtained (Fig. 3a). When added at 0 h, 300 μM BDSF or trans-BDSF reduced biofilm formation by approximately 90%.

The addition of 3, 30 and 300 μM farnesol and DSF at 1 h after cell attachment showed almost no effect on biofilm formation. The XTT readings showed little fluctuation when compared with the control readings. In contrast, BDSF and trans-BDSF, at 30 μM, had a moderate but significant effect on biofilm formation and successfully prevented germination of the adherent yeast cells, whilst at concentrations of 300 μM, biofilm formation was reduced significantly by approximately 60% (Fig. 3b).

When added at 2 h after cell attachment, only BDSF and trans-BDSF at 30 μM had a significant inhibitory effect on cell growth and morphology. The effects were less noticeable than when added at 0 and 1 h after cell attachment. At 300 μM, both BDSF and trans-BDSF reduced biofilm production by approximately 40% (P<0.05; Fig. 3c).

When added at 4 h after cell attachment, at 3 and 30 μM, none of the four drugs had any significant inhibitory effect.
on biofilm formation ($P>0.05$), and *C. albicans* formed a biofilm that closely resembled the control biofilm. All XTT readings were similar to those of the controls. However, at a concentration of 300 μM, BDSF and *trans*-BDSF reduced biofilm formation significantly by approximately 30% (Fig. 3d).

For pre-formed *C. albicans* biofilms, the XTT readings remained steady for all four drugs at all the concentrations tested (data not shown). Microscopic examination revealed only a small reduction in biofilm formation, indicating that *C. albicans* biofilms display high levels of resistance to these drugs. An approximate 10% reduction in pre-formed *C. albicans* biofilms was achieved by BDSF and *trans*-BDSF at concentrations of 300 μM.

The colorimetric assay and microscopy indicated that farnesol and DSF were only active at high concentrations; in contrast, BDSF and *trans*-BDSF proved to be highly active in a dose- and time-dependent manner, reflected by the decrease in $A_{490}$ values with increasing concentrations of BDSF and *trans*-BDSF (Fig. 3). Visualization of the biofilm structure revealed that, compared with the untreated biofilm control, the greatest reduction in biofilm constituents was caused by BDSF and *trans*-BDSF, followed by farnesol, whilst DSF caused the smallest reduction (Fig. 2). These results demonstrated that BDSF and *trans*-BDSF not only inhibited yeast-cell growth and germ-tube formation, but also reduced the number of adhering cells and biofilm development.

**Expression analysis with real-time RT-PCR**

To investigate whether the effect of blocking biofilm formation was the result of inhibition of hyphae formation, real-time RT-PCR was employed to elucidate the effects of BDSF and *trans*-BDSF on expression of the hyphae-specific genes *HWP1* and *ALS3* (Argimón *et al.*, 2007; Green *et al.*, 2004; Hoyer *et al.*, 2008; Staab & Sundstrom, 1998; Staab *et al.*, 2004). Prior studies have shown that *HWP1* is not expressed during yeast-phase growth but is expressed strongly in germ tubes and hyphal surfaces, implicating this gene in filamentation and biofilm formation. Hyphal development is thought to be affected by differential expression of morphogenic functions in response to external signals. Examining the influence of these signals on *HWP1* expression may elucidate several facets of biofilm development. The agglutinin-like sequence (ALS) family of *C. albicans* genes encodes a set of differentially regulated cell-surface glycosylphosphatidylinositol-anchored glycoproteins, which promote adherence (Green *et al.*, 2004; Hoyer *et al.*, 2008). *ALS3* is expressed specifically during hyphal development in *C. albicans* (Argimón *et al.*, 2007).

Real-time RT-PCR was utilized to investigate changes in the expression levels of the *HWP1* and *ALS3* genes. It showed that both *HWP1* and *ALS3* were downregulated in a dose-dependent way after treatment with BDSF and *trans*-BDSF. At a concentration of 30 μM, BDSF inhibited the expression of *HWP1* and *ALS3* by 59.53 and 67.98%, respectively; at 60 μM, these genes were inhibited by 88.46%.
and 89.33%, respectively. trans-BDSF behaved somewhat differently; at 30 μM HWP1 and ALS3 were downregulated by 48.64 and 51.43%, respectively, and by 67.24 and 81.07% at 60 μM (Fig. 4). Combined with the effect that these two drugs had on C. albicans germ-tube formation and yeast-cell growth, this result seemed to confirm that, at low concentrations, BDSF and trans-BDSF can block C. albicans biofilm formation by interfering with the morphological switch, whilst at higher concentrations, they can inhibit growth and germ-tube formation.

**DISCUSSION**

Biofilms form a protective niche for micro-organisms where they can avoid the effects of antibiotics and create a source of persistent infection (Chandra et al., 2001). Biofilms therefore make clinical care more challenging. Biofilms have unique characteristics compared with planktonic cells (Ramage et al., 2001a, b; Stephens, 2002). Biofilms are highly organized (Costerton, 1995) and are not disorderly amalgamated communities of cells. The importance of C. albicans biofilm formation and its role in infections have been well documented (Ramage et al., 2002b). Effective antifungal drugs that can prevent and destroy C. albicans biofilm are urgently required. The cells in microbial biofilms release chemical compounds in a concerted reaction that reach threshold densities. These signal molecules initiate cellular differentiation events (Davies et al., 1998; Miller & Bassler, 2001; Parsek & Greenberg, 1999; Ramage et al., 2002a; Singh et al., 2000). Farnesol, a quorum-sensing molecule from C. albicans (Hornby et al., 2001), blocks filamentation and inhibits biofilm formation by suppressing the dimorphic switch (Ramage et al., 2002a). As a prerequisite for its quorum-sensing activity, it is necessary that farnesol does not alter C. albicans growth and growth rate (Henriques et al., 2007). Therefore, farnesol might be useful as a novel therapeutic antifungal compound for immunocompromised patients because it will not upset the natural resident flora.

Cross-kingdom activity has been proposed for fatty acid messengers. Such activity leads to inhibition of conversion from yeast to hyphae in C. albicans (Wang et al., 2004). This proposal is further supported by the observation that cis-rather than trans-2-decenoic acid can induce the dispersion of C. albicans biofilms (Davies & Marques, 2009). BDSF is structurally related to farnesol and similar to DSF, both of which have been shown to inhibit germ-tube formation in C. albicans (Hogan et al., 2004; Wang et al., 2004). This suggests that these short-chain cis-2-monounsaturated fatty acids probably have deep evolutionary roots. Their relationship and biological function is complex, involving various metabolic pathways. The precise mechanisms of the resistance function associated with microbial morphogenesis and biofilms of C. albicans remain unclear.

As BDSF acts by preventing the formation of the hyphal form of C. albicans, the main aim of this work was to investigate the effect of BDSF and trans-BDSF on yeast growth rate and biofilm formation. We found that, at low concentrations (3 μM), trans-BDSF and BDSF inhibited germ-tube formation in approximately 70% of the cells, and this was similar to the effect of farnesol and DSF at much higher concentrations (300 μM). Interestingly, 300 μM farnesol and DSF had no effect on the yeast-cell growth rate, whilst, at the same concentration, BDSF and trans-BDSF had a large and detrimental effect on yeast-cell growth. At lower concentrations of BDSF and trans-BDSF, the morphology of the biofilms changed in a dose-dependent manner, consistent with the results of a prior study (Boon et al., 2008). A previous report showed that trans-2-decenoic acid dispersed the C. albicans biofilm only at millimolar concentrations (Davies & Marques, 2009). In the present study, the inhibitory effects of BDSF and trans-BDSF on biofilm formation showed a dose- and time-dependent trend. Compared with farnesol and DSF, the effects of BDSF and trans-BDSF were much stronger. Only high concentrations of farnesol and DSF, added to the C. albicans suspension at 0 h after attachment, reduced biofilm formation. BDSF and trans-BDSF reduced biofilm formation when added at 0, 1, 2 and even 4 h after attachment, with 300 μM BDSF and trans-BDSF reducing biofilm formation by approximately 30% at the last time point. Prior studies using 300 μM farnesol have demonstrated its inhibitory effect on pre-formed biofilm when added at 24 h after biofilm formation (Ramage et al., 2002a). A similar trend was not detected in the present study. The main difference between the four drugs in blocking C. albicans biofilm formation was that, whilst farnesol and DSF inhibit germ-tube germination, BDSF and trans-BDSF inhibit yeast-cell growth and germ-tube germination at high concentrations and inhibit germ-tube germination only at low concentrations. Both trans-BDSF and BDSF were better inhibitors of biofilms than farnesol.

![Fig. 4. Comparison of HWP1 and ALS3 expression levels in biofilms treated with BDSF or trans-BDSF relative to expression of the housekeeping gene ACT1.](image-url)
and DSF. This result may provide an alternative anti-infective strategy to deal with *C. albicans* biofilm infections.

Adherence is a critical property for biofilm cells, and multiple adhesion molecules could function as successful biofilm formers. In this study, the adherence times allowed before the drugs were added played a crucial role in the ability of the drugs to inhibit biofilm formation. We have begun a study to investigate the mechanism of action of BDSF on adherence in the yeast phase. The observation that BDSF is a highly potent inhibitor of *C. albicans* biofilm formation raises intriguing questions about the molecular mechanism of signal interference in early biofilm formation and the potential role of this signal in competition with internal signalling systems.

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