Full Paper

Suppression of the pathogenicity of *Candida albicans* by the quorum-sensing molecules farnesol and tryptophol

(Received October 9, 2018; Accepted December 29, 2018; J-STAGE Advance publication date: June 18, 2019)

Pantira Singkum,¹ Watcharamat Muangkaew,¹ San Suwanmanee,¹ Potjaman Pumeesat,²
Thanwa Wongsuk,³ and Natthanej Luplertlop^{1,*}

¹ Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand

² Department of Medical Technology, Faculty of Science and Technology, Bansomdejchaopraya Rajabhat University, Bangkok 10600, Thailand

³ Department of Clinical Pathology, Faculty of Medicine, Vajira Hospital, Navamindradhiraj University, Bangkok 10300, Thailand

This study examines the ability of the quorum-sensing molecules (QSMs) farnesol and tryptophol to induce programmed cell death of the pathogenic fungus *Candida albicans*, to alter the expression of apoptosis-related genes, and to reduce the pathogenicity and virulence of *C. albicans* in *Galleria mellonella*. Our results showed that both farnesol and tryptophol inhibited *C. albicans* germ tube formation. In the QSM-treated group, the expression levels of the apoptosis genes increased, whereas the expression level of the anti-apoptosis gene decreased. Further, pretreatment of *C. albicans* with tryptophol or farnesol prior to *G. mellonella* larval infection significantly enhanced host survival compared with larvae infected with untreated *C. albicans*. Thus, farnesol and tryptophol may trigger apoptosis of *C. albicans* *in vitro* and reduce the virulence of *C. albicans* *in vivo*. Although further study is needed to identify the precise mechanisms underlying the antifungal properties of farnesol and tryptophol, these results suggest that QSMs may be effective agents for controlling fungal infection.

Key Words: *Candida albicans*; *Galleria mellonella*; germ tube; quorum-sensing molecules; tryptophol

Introduction

Candida albicans is a dimorphic fungus that grows as both yeast and in filamentous forms. It is present as normal flora in various organisms, such as the oral, vaginal,

and genital tracts of warm-blooded animals, including humans. However, *C. albicans* is also an important opportunistic pathogen in immunocompromised hosts (Alangaden, 2011; Armstrong, 1993; Odds, 1996; Wenzel, 1995). One of the crucial factors for the virulence of *C. albicans* is its ability to switch from yeast to hyphae (Lo et al., 1997; Mayer et al., 2013; Mitchell, 1998) because the hyphal form can adhere and penetrate tissues more readily than the yeast form (Brennan, 2002; Grubb et al., 2008; Hornby et al., 2004; Shen et al., 2008; Wang and Lin, 2012). The first stage in this transition is the formation of a germ tube, which is triggered by interaction with the host cell and is dependent on factors, such as serum, pH, temperature, and quorum-sensing molecules (QSMs) (Grubb et al., 2008; Wang and Lin, 2012).

QS is a mode of microbial cell-cell communication that depends on cell density and serves to regulate fungal behaviors, such as biofilm formation, competence, and bioluminescence (Grubb et al., 2008; Shen et al., 2008; Wang and Lin, 2012). At the molecular level, these changes require the release of hormone-like QSMs (Nealson and Hastings, 1979). QSMs have been the subject of studies dating back to the 1960s and 1970s on bacteria, such as *Streptococcus pneumoniae* and bioluminescent marine *Vibrio* species, respectively (Hastings and Nealson, 1977; McMillan et al., 2015; Nealson and Hastings, 1979). In eukaryotes, the QSM farnesol (FOH) was first isolated from *C. albicans*. In addition, other QSMs have been found in *C. albicans*, such as tyrosol, phenylethanol, and tryptophol (TOH). In fungi, QSMs are important regulators of morphogenesis, biofilm development, and cell population density control (Alem et al., 2006; Lindsay et al., 2012; Wongsuk et al., 2016). The QSM FOH has been

*Corresponding author: Natthanej Luplertlop, Department of Microbiology and Immunology, Faculty of Tropical Medicine, Mahidol University, Bangkok 10400, Thailand.

Tel: +66 (0) 2306 9172 Fax: +66 (0) 2643 5583 E-mail: natthanej.luplertlop@mahidol.ac.th

None of the authors of this manuscript has any financial or personal relationship with other people or organizations that could inappropriately influence their work.

Table 1. Primer sequences for qPCR analyses of *CARD-9*, *NOXA*, and *Bcl-2* expression.

Primers	Forward	Reverse
<i>CARD-9</i>	5'-TCCGACCTGGAAGATGGCTCAC-3'	5'-CAGAGCTGCAAAGGGCTGTTTC-3'
<i>NOXA</i>	5'-GCTGGAAGTCGAGTGTGCTA-3'	5'-CCTGAGCAGAAGAGTTTGA-3'
<i>Bcl-2</i>	5'-TTGTGGCCTTCTTTGAGTTCGGTG-3'	5'-GGTGCCGGTTCAGGTACTCAGTCA-3'
β -tubulin	5'-ATAATGTTTCAGACCGCCCTCTGCT-3'	5'-GACCGATGTGGAATTGCCACAAA-3'

shown to trigger programmed cell death and inhibit yeast-to-hyphae switching (Hornby et al., 2004; Lindsay et al., 2012), whereas TOH has been found to regulate the germination of *Aspergillus* sp., adhesion and sliding motility in *Debaryomyces hansenii*, and hyphal growth of *Candida* spp. (Albuquerque and Casadevall, 2013; Alem et al., 2006; Lindsay et al., 2012).

Apoptosis is a form of programmed cell death triggered by various stressors. Mitochondrial dysfunction is a key trigger for apoptosis. Genes such as *CARD-9* (encoding caspase recruitment domain family member 9) and *NOXA* (encoding phorbol-12-myristate-13-acetate-induced protein 1) act to facilitate apoptosis in response to fungal infection and DNA damage, respectively. Both genes have been widely studied in eukaryotes (Bouchier-Hayes, 2002), but contributions to fungal apoptosis remain unclear. Other seminal regulators of mitochondrial-dependent apoptosis include B-cell lymphoma-2 (*Bcl-2*) family proteins, which may either induce or inhibit apoptosis (Bertin et al., 2000; Bouchier-Hayes, 2002; McIlwain et al., 2013).

The greater wax moth *Galleria mellonella* (order Lepidoptera, family Pyralidae) is a globally distributed species commonly used as an alternative model to study microbial infections and toxicity (Beth et al., 2010) in place of mice and rabbits (Arvanitis et al., 2013; Hoffmann et al., 1999). Robust cellular and humoral immune responses make *G. mellonella* larvae an advantageous model for fungal infection studies (Fallon and Sun, 2001; Hoffmann et al., 1999; Mowlds et al., 2008; Vogel et al., 2011; Wojda, 2016; Wojda et al., 2009).

In the present study, we examined FOH- and TOH-induced apoptosis in *C. albicans* and the effects of these QSMs on fungal pathogenesis in *G. mellonella*.

Materials and Methods

***G. mellonella* selection for infection.** *G. mellonella* larvae were obtained from Jerry® Wax Worms (Thailand). Larvae were reared on a media containing bran, all-purpose flour, dried bakery yeast, liquid honey, and glycerol at room temperature in plastic rearing boxes with grids prior to experiments. Last-stage larvae were selected when they stopped feeding and started to produce a thin silk body cover (approximately 2 months after their eggs hatched). At this stage, their weight was 200–400 mg and cuticles were lightly colored without gray areas (Beth et al., 2010; Fallon and Sun, 2001; Hoffmann, 1999; Mowlds et al., 2008; Tsai et al., 2016; Vogel et al., 2011; Wojda, 2016; Wojda et al., 2009). The larvae were then transferred to sterile Petri dishes for 24 h without feeding, and the nascent protective cocoon was removed.

Yeast preparation. *C. albicans* strain ATCC90028 was

obtained from the American Type Culture Collection (ATCC) and cultured in Yeast Peptone Dextrose Broth (YPDB) (Difco™, USA) at 30°C overnight, conditions conducive to the development of the yeast form. Yeast cells were collected, washed twice with phosphate-buffered saline, counted with a hemocytometer, and adjusted to 5×10^7 cells/ml in YPDB.

QSMs-treated *C. albicans* preparation. Farnesol (FOH) and Tryptophol (TOH) (Sigma-Aldrich, USA) was obtained as a 1 M stock solution and then diluted to obtain a 1,000 μ M working stock solution in 100% (vol/vol) methanol. The FOH and TOH were prepared with YPDB plus 20% fetal bovine serum (FBS) by the 300 μ M and 200 μ M concentration. *C. albicans* yeast cells were adjusted to 5×10^7 cells/ml under each condition and incubated for 2, 6 and 24 h at 37°C.

QSMs treatment and *G. mellonella* infection. Moth larvae were divided into nine treatment groups, each with nine larvae. As anesthesia, larvae were cooled in Petri dishes at 5–8°C for 1 day and then injected with a *C. albicans* suspension or vehicle. Briefly, moth larvae were held carefully by the thumb, index finger, and middle finger, and the injection area cleaned using 70% ethanol. *C. albicans* suspension was collected at each time point and injected into the larvae immediately. Larvae were injected by 10 μ l of the injected reagent at the second proleg area according to the treatment group: (1) Non-injection control, (2) Vehicle (YPDB)-injected uninfected control, (3) *C. albicans* at 5×10^7 cells/ml, (4) 200 μ M TOH-treated *C. albicans* at 5×10^7 cells/ml, (5) 300 μ M FOH-treated *C. albicans* at 5×10^7 cells/ml, (6) 200 μ M TOH alone, (7) 300 μ M FOH alone, (8) 0.03% absolute ethanol (TOH vehicle), and (9) 0.03% absolute methanol (FOH vehicle). The indicated solution was injected into the larval hemocoel via the last proleg using a 30G single-use sterile syringe. If the initial penetration was incorrect, hemolymph leaked out from the injection site, but this leakage stopped within a minute. Following injection, the needle was carefully removed, and the larvae were incubated in Petri dishes without food at 37°C in the dark for survival analysis.

***G. mellonella* survival time and melanization.** The survival of larvae was checked daily for up to 7 days post-injection. Larvae were poked with forceps to check the response to physical stimuli, and the cuticle color was monitored. Dead larvae lacked movement and turned completely black. The surviving larvae were used for further studies. The endpoint of this experiment was the death of all larvae in the group or the transition into pupa.

Fixation and sectioning. Dead larvae were fixed by in-

Fig. 1. Proportions of surviving larvae after injection of the indicated *C. albicans* dose.

jecting 150 μ l of 10% formalin into the hemocoel via the last proleg, followed by immersion in 1 ml of freshly prepared 10% formalin. The fixative agent was renewed at 1, 5, and 24 h, and fixation continued for 1 week. The fixed larvae were then transferred into cassettes and placed in an automated tissue processor. The tissue was dehydrated in a graded ethanol series (70%, 80%, 90%, and 100%), immersed in xylene, and embedded with paraffin. Paraffin blocks were stored to harden overnight at -20°C and then cut into approximately 5- μ m sections using a microtome. Sections were stained with hematoxylin and eosin (H&E) or Gomori methenamine silver (GMS), mounted on slides, and examined under 40 $\times$ and 100 $\times$ magnification using a Nikon Eclipse E200 microscope (Japan).

QSMs treatment and CARD-9, NOXA, and Bcl-2 gene expression. Yeast cells were counted and adjusted to 1×10^4 cells/ml in YPDB. Expression was examined under three conditions: YPDB plus 20% fetal bovine serum (FBS) alone (control group), YPDB + 20% FBS + 200 μ M TOH, and YPDB + 20% FBS + 300 μ M FOH and the cells were harvested at 2, 6, and 24 h post treatment. RNA was extracted using TRIzol[®] reagent (TRIzol[™], USA) according to the manufacturer's recommendation and real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR) was performed using the KAPA SYBR[®] FAST One-Step qRT-PCR Master Mix (2X) Kit (Kapa Biosystems, USA). Primer sequences of each gene are shown in Table 1. The relative gene expression of the apoptosis-related genes *CARD-9*, *NOXA*, and *Bcl-2* were normalized with beta tubulin.

Acridine Orange/Ethidium Bromide (AO/EB) staining to detect apoptosis of QSMs-treated *C. albicans*. This work was carried out under the following conditions: YPDB plus 20% fetal bovine serum (FBS) alone (control group); YPDB + 20% FBS + 200 μ M TOH; and YPDB + 20% FBS + 300 μ M FOH. In the case of each condition, yeast cells were adjusted to 5×10^7 cells/ml and incubated for 2, 6, and 24 h. Each of the samples were mixed by vortex and transferred to a 15 ml centrifuge tube. The cells were centrifuged for 5 minutes at 2500 rpm. The supernatant was removed and then resuspended with 1 ml of sterile PBS. Yeast cells were transferred to a microcentrifuge tube and washed with sterile PBS by using a centrifuge for 10 minutes at 2500 rpm. Twenty microliters of cell suspension was mixed with 2 μ l of AO/EB solution. Each sample was mixed prior to microscopy

Fig. 2. The different characteristics of *G. mellonella* larvae before and after being infected with *C. albicans*.

A. the characteristics of *G. mellonella* used in this study, uninfected larvae were alive and lightly colored without grey markings on the cuticle; whereas B. larvae infected with 1×10^8 cell/ml *C. albicans* showed a clear spot and tail line melanization (arrows). Melanization is the process of melanin synthesis and deposition, which occurs to encapsulate pathogens at the wound site followed by hemolymph coagulation and opsonization normally starts with characteristic black spots and tail line on the larvae skin. Melanization correlates with infection of the larvae that can also be used as a marker for disease in *G. mellonella* larvae.

and evaluated immediately. Ten microliters of cell suspension were placed onto a glass slide, covered with a glass coverslip, and examined using a fluorescence microscope, using a fluorescein filter and a 40 $\times$ magnification.

Ethics approval of *G. mellonella* studies. All applicable international, national, and institutional guidelines for the care and use of animals were followed, and the study was approved by the research ethics committee of Mahidol University, Thailand (approval certificate number MU-IACUC 2018/015).

Statistical analyses. All experiments was done in triplicate. The data was presented as the mean $\pm$ SD to compare the difference between groups by using Mann-Whitney U test. A P-value of <0.05 was considered statistically significant.

Results

Infectious dose dependence of *G. mellonella* larval mortality

Trial infection experiments were performed to determine an optimal (mid-range) infectious dose of *C. albicans* in the *G. mellonella* model. *G. mellonella* larvae were injected with 10 μ l of 10^9 , 10^8 , 10^7 , or 10^6 *C. albicans* strain ATCC90028 cells/ml, and mortality rates were determined 0–72 h post-infection. Larvae exhibited gray patches on the cuticle within 1–2 h after infection with *C. albicans*. Infection with 10^9 cells/ml resulted in 100% mortality by 1 day post-infection, 10^8 cells/ml induced 60% mortality by 1 day and 100% by 2 days post-infection, 10^7 cells/ml induced 90% mortality at 72 h post-infection, and 10^6 cells/ml induced approximately 40% mortality at 72 h post-infection (Fig. 1). Fungal doses of 10^7 and 10^8 cells/ml also induced clear melanization (Fig. 2), but the lower dose led to only 50% death in 48 h. Therefore, we chose a *C. albicans* dose of 5×10^7 cells/ml to inoculate *G. mellonella* for studies on QSM-mediated protection to obviate ceiling and floor effects.

Fig. 3. Proportions of surviving larvae in each treatment group.

Fig. 4. Melanization of *G. mellonella* after experimental treated.

A. *G. mellonella* larvae appeared melanized a few minutes after *C. albicans* injection. The arrows show melanized accumulation sites manifested as black spots (blue arrow) and a tail line (red arrow). B. and C. *G. mellonella* larvae treated with QSMs show no different morphology changes.

Fig. 5. Histopathological changes in infected larvae revealed by H&E staining.

A. Uninjected larvae, B. larvae injected with untreated *C. albicans*, C. larvae injected with FOH-treated *C. albicans*, and D. larvae injected with TOH-treated *C. albicans*. Images were acquired at 100× under a light microscope.

Pretreatment of *C. albicans* with TOH or FOH reduces pathogenicity in the *G. mellonella* infection model

We then compared the survival rates of *G. mellonella* larvae infected with 5×10^7 cells/ml untreated or QSM-treated *C. albicans* to uninfected, vehicle, and QSM-only as a control. There was no evidence of animal death in both QSMs alone and *C. albicans* growth medium. Three larvae injected with untreated *C. albicans* died by 1 day post-infection (67.67% survival). Mortality increased sig-

Fig. 6. Histopathological changes in infected larvae revealed by GMS.

A. Uninjected larvae, B. larvae injected with untreated *C. albicans*, C. larvae injected with FOH-treated *C. albicans*, and D) larvae injected with TOH-treated *C. albicans*. Images were acquired at 100× under a light microscope.

Fig. 7. Apoptosis gene expression of *G. mellonella* after the experimental treated.

The relative expression levels of *CARD-9* (A), *NOXA* (B), and *Bcl-2* (C) in untreated and QSM-treated *C. albicans*.

nificantly ($p < 0.05$) thereafter, with the daily survival rate falling progressively from 66.67% on day 2 to 55.56% on day 3, 33.33% on days 4 and 5, 22.22% on day 6, and finally to 0% on day 7. This control mortality rate was significantly reduced when *C. albicans* was pretreated with FOH (daily survival rates of 100%, 100%, 100%, 100%, 88.89%, 77.78%, and 77.78%) or TOH (100%, 100%, 100%, 100%, 100%, 100%, 88.89%, and 88.89%). Neither the uninjected control group nor the media-injected control groups exhibited any mortality (100% survival on day 7). Similarly, neither QSM alone nor the solvents (ethanol for TOH and methanol for FOH) induced mortality (Fig. 3). Therefore, TOH and FOH appear to reduce the pathogenicity of *C. albicans* in *G. mellonella*.

Infection was also associated with impaired motility, surface lesions, and skin melanization. In moth larvae infected with 5×10^7 cells/ml untreated *C. albicans*, the

Fig. 8. Apoptosis induction of *C. albicans* at 24 h by using AO/EB double-staining under fluorescent microscopy.

A. Negative control group (*C. albicans* treated with YPDB plus 20% fetal bovine serum. B. FOH treated group (*C. albicans* treated with YPDB plus 20% FBS and 300 μ M FOH). C. TOH treated group (*C. albicans* treated with YPDB plus 20% FBS and 200 μ M TOH). Images were acquired at 100 $\times$ under a fluorescent microscope. L; live cells, N; indicate necrotic cells, A; indicate apoptotic cells, y; yeast cell, b; budding yeast, ps; pseudohyphae, and t; true hyphae.

cuticular color changed from lightly colored to dark within 1–2 h of injection, because of the accumulation of melanin, and eventually turned completely black near the time of death. Melanization initially occurred in the dorsal portion as a spot and tail line (Fig. 4A). In contrast, both uninjected and vehicle-injected control moth larvae remained lightly colored. Consistent with the mortality results, larvae injected with TOH-treated *C. albicans* (Fig. 4B), FOH-treated *C. albicans* (Fig. 4C), 200 μ M TOH alone, 300 μ M FOH alone, 0.03% absolute ethanol, or 0.03% absolute methanol showed no marked color change (data not shown).

Histopathological examination of infected larval tissue

To elucidate the potential underlying mechanisms for larval death and survival, we examined histopathological changes associated with pathogen infection and ensuing melanization in paraffin-embedded sections stained with H&E or GMS. Healthy *G. mellonella* have an open circulatory system comprising many blood vessels projecting between muscle and adipose or connective tissue in a single-layer arrangement. The middle body between the second to ninth segment contains the digestive system (midgut), and the body is normally surrounded by a lightly colored cuticle. Nuclei were stained dark blue or purple by hematoxylin, whereas the cytoplasm was stained red or pink by eosin because of the presence of proteins. Tissues from uninfected moths showed normal cellular arrangements, and hemocytes did not accumulate at the vehicle-injection site (Fig. 5A). No changes were observed 1 and 2 days post-infection (picture not shown). By day 3 post-infection, however, *C. albicans* had spread throughout the fat bodies of *G. mellonella* larvae. Yeast and filamentous fungi were observed in gut sections from larvae injected with untreated *C. albicans*, primarily in clusters. The majority of hemocytes were in tightly packed aggregates (nodules) with signs of melanization (Fig. 5B, black circles). In contrast, these aggregates were absent in sections from control groups. Infected moth larvae also exhibited mid-gut damage not observed in sections from control groups, whereas other tissues (fat bodies and muscles) appeared undamaged. Pigmented nodules were also present in infected larvae, and fungal cells were associated with these nodules (Fig. 5B, black circle).

Larvae injected with TOH- or FOH-treated *C. albicans* exhibited fewer areas of infection than those exhibited by larvae injected with untreated *C. albicans*, and larvae in these TOH- and FOH-treated groups exhibited generally normal histological characteristics (Figs. 5C and 5D).

H&E cannot distinguish the shape of *C. albicans* within infected tissue, so other sections were examined by GMS, a popular staining method for screening fungal infection and particularly useful for staining fungal carbohydrates. The cell walls of infected fungi were stained brown to black on a green background. We observed clusters of black-stained fungal cells on a green background that colocalized with melanization nodule sites. Most yeast were round or ovoid and about 3–5 μ m in diameter. Budding was observed in some cells. Pseudohyphae about 3–5 μ m in width were also found, which looked like mold, and other yeast cells exhibited a narrow area at the end. Some cells had transformed to true hyphae characterized by a straight rod shape. These hyphae were arranged in parallel with no constriction (Fig. 6).

CARD-9, NOXA, and Bcl-2 gene expression by *C. albicans*

In case of both FOH and TOH treatment conditions, the expression levels of *CARD-9* and *NOXA* significantly increased, whereas *Bcl-2* expression significantly decreased compared with the untreated controls (Fig. 7). The highest *CARD-9* expression was observed after 2 and 6 h of TOH treatment. *CARD-9* expression was initially lower in FOH-treated *C. albicans* than in TOH-treated *C. albicans* but increased to a higher level at 24 h (Fig. 7A). The expression level of *NOXA* was also elevated after 6 and 24 h of FOH and TOH treatment compared with that of untreated *C. albicans* (Fig. 7B). Conversely, *Bcl-2* expression was reduced under both test conditions at 24 h and was the lowest at each time point in TOH-treated cells, while the *Bcl-2* trend showed slightly up-regulated in FOH treated *C. albicans* at 24 h (Fig. 7C).

AO/EB double-staining and fluorescent microscopy

Yeast cells were stained by AO/EB 2, 6, and 24 h after FOH and TOH were treated. Dual staining was examined under a fluorescent microscope. The results showed no significant apoptosis detected at 2 and 6 h (data not shown).

At 24 h, apoptotic cells marked by crescent-shaped or granular yellow-green AO nuclear staining, and orange nuclear EB staining, were detected in FOH and TOH treated groups (Figs. 8B and 8C) when compare with the negative control group (Fig. 8A). In the case of pathogen forms, the highest amount of yeast form showed in the TOH treated group (Fig. 8C), whereas the FOH treated and negative control group showed several forms including yeast, budding yeast, pseudohyphae and true hyphae (Fig. 8A). Staining was restricted irregularly within the cells. With FOH and TOH usage concentrations, the apoptotic cells (early-stage) and necrosis cells (late-stage) appeared as a yellow-green fraction and orange nuclear staining in cells.

Discussion

QSMs have been extensively studied in both yeast and mold species, such as *C. dubliniensis*, *A. niger*, *A. nidulans*, and *Fusarium graminearum*, but most of these studies have focused on fungal morphogenesis (yeast-to-hypha formation) and the germination process of macroconidia (Lo et al., 1997). In addition, QSMs have been implicated in programmed cell death or fungal apoptosis, a process that may also markedly influence fungal pathogenicity. In the present study, we examined two QSMs, TOH and FOH, because previous studies have demonstrated that both reduce mold formation (filamentation) in *Aspergillus* spp. (Chen and Fink, 2006; Hornby et al., 2004; Lindsay et al., 2012; Nickerson et al., 2006; Wongsuk et al., 2016) by inhibiting germ tube formation, resulting in a slower change in the shape of the germ (Hornby et al., 2004). However, the functions of these QSMs in the regulation of fungal pathogen virulence have not yet been investigated.

Our results show that *C. albicans* treated with FOH or TOH is less virulent when injected into *G. mellonella* than untreated *C. albicans*, as evidenced by the dramatically enhanced survival rate. Indeed, the survival rate of *G. mellonella* larvae injected with untreated *C. albicans* was 0% within 7 days compared with 77.78% for *G. mellonella* infected with FOH-treated *C. albicans* and 88.89% for *G. mellonella* infected with TOH-treated *C. albicans*. FOH was previously shown to decrease the expression of *C. albicans* pescardillo homolog (PES1), a gene required for yeast-to-hyphae switching in *C. albicans*, suggesting that reduced hypha formation contributes to the lower pathogenicity of QSM-treated *C. albicans*. These effects of TOH treatment on *C. albicans* pathogenicity in *G. mellonella* have not been previously reported, and several studies on FOH applied to the host following *C. albicans* injection (Fuchs et al., 2012; Jacobsen, 2014; Li et al., 2013; Mesa-Arango et al., 2013; Pappas et al., 2009; Scorzoni et al., 2013). Our study is the first to demonstrate a TOH-mediated reduction in *C. albicans* pathogenicity and to implicate apoptosis induction in this effect.

The primary immune responses of *G. mellonella* against *C. albicans* are nodulation and melanization (Wojda, 2016). Melanization comprises the synthesis and deposition of melanin to encapsulate pathogens at the wound site, typically followed by hemolymph coagulation and

opsonization. The early melanization response in *G. mellonella* is manifested by spotting and tail lines on the cuticles, which were observed within hours following an injection of untreated *C. albicans*. In contrast, no such changes were observed in the control groups or in *G. mellonella* injected with FOH- or TOH-treated *C. albicans*. These coloration changes in moths injected with untreated *C. albicans* were correlated with histopathological changes revealed by H&E, which showed nodule formation and associated melanin pigmentation. The reduction of melanization in *G. mellonella* injected with QSM-treated *C. albicans* is consistent with reduced fungal virulence.

In sections of infected tissue, *C. albicans* presented in both yeast and filamentous forms as evidenced by GMS, whereas FOH- and TOH-treated *C. albicans* presented predominately as yeast. These results are consistent with the notion that both QSMs tested suppress virulence by inhibiting yeast-to-hyphae transition. Indeed, the development of hyphae or pseudohyphae is strongly correlated with the virulence of *Candida* species, such as *C. albicans*, *C. krusei*, and *C. tropicalis*.

FOH is known to induce apoptosis of *C. albicans*, although this is the first report on the apoptotic effect of TOH. As in mammals, apoptotic pathways in fungi appear to depend on mitochondrial dysfunction. FOH-induced apoptosis in *C. albicans* occurs via the induction of caspases, production of Reactive Oxygen Species, and disruption of mitochondrial membrane integrity. The expression levels of *CARD-9* and *NOXA* were elevated by both FOH and TOH. The *CARD-9* protein is associated with Bcl-10, a positive regulator of apoptosis and activation of the Nuclear Factor-Kappa B stress pathway. Additionally, the pro-apoptotic *Bcl-2* family protein *NOXA* can be induced at the mitochondrial membrane and can directly regulate numerous other *Bcl-2* family proteins in the cytoplasm (Bertin et al., 2000; McIlwain et al., 2013). In both FOH- and TOH-treated groups, the expression of *Bcl-2* decreased. Although *Bcl-2* can induce or inhibit apoptosis depending on cellular context, the major function appears to be anti-apoptotic (Bertin et al., 2000). Moreover, we confirm results by using Acridine orange/Ethidium bromide staining to determine apoptosis. Acridine orange is a vital dye and will stain both live and dead cells. Ethidium bromide will stain only cells that have lost membrane integrity. Live cells will appear uniformly green. Apoptotic cells will stain green and contain bright green dots in the nuclei as a consequence of chromatin condensation and nuclear fragmentation. Additionally, apoptotic cells will also incorporate ethidium bromide and therefore stain orange, cells will show condensed and often fragmented nuclei. From our results, *C. albicans*-apoptotic cells were induced by QSMs when incubated for 24 h. Therefore, FOH and TOH appear to induce apoptosis in *C. albicans*. Further studies are required to confirm that activation of the apoptotic pathway reduces the virulence of *C. albicans*.

We have demonstrated that QSMs can reduce the virulence of *C. albicans* in *G. mellonella*. Further, the present study demonstrates the utility of *G. mellonella* as an alternative fungal infection model. This model may be ap-

plicable for the histopathological study of other fungal infections and treatment strategies.

Author Contributions

Conceived of or designed study: Natthanej Luplertlop and Pantira Singkum

Performed research: Natthanej Luplertlop, Pantira Singkum, Watcharamat Muangkaew, San Suwanmanee, Potjaman Pumeesat and Thanwa Wongsuk

Analyzed data: Natthanej Luplertlop and Pantira Singkum

Contributed new methods or models: Pantira Singkum, San Suwanmanee and Watcharamat Muangkaew

Wrote the paper: Pantira Singkum and Natthanej Luplertlop

Acknowledgments

This study was supported by a Thesis Grant for Doctoral Degree Student of The National Research Council of Thailand (NRCT) (2016) and Tropical Medicine grants 2014, Faculty of Tropical Medicine, Mahidol University.

References

- Alangaden, G. (2011) Nosocomial fungal infections: epidemiology, infection control, and prevention. *Infect. Dis. Clin. N. Am.*, **25**, 201–225.
- Albuquerque, P. and Casadevall, A. (2013) Quorum sensing in fungi—a review. *Med. Mycol.*, **50**, 337–345.
- Alem, M. A., Oteef, M. D., Flowers, T. H., and Douglas, L. J. (2006) Production of tyrosol by biofilms and its role in quorum sensing and biofilm development. *Eukaryot Cell*, **5**, 1770–1779.
- Armstrong, D. (1993) History of opportunistic infection in the immunocompromised host. *Clin. Infect. Dis.*, **17**, S318–S321.
- Arvanitis, M., Glavis-Bloom, J., and Mylonakis, E. (2013) Invertebrate models of fungal infection. *Biochim. Biophys. Acta*, **1832**, 1378–1383.
- Bertin, J., Guo, Y., Wang, L., Srinivasula, S. M., Jacobson, M. D. et al. (2000) CARD9 is a novel caspase recruitment domain-containing protein that interacts with BCL10/CLAP and activates NF- κ B. *J. Biological. Chem.*, **275**, 41082–41086.
- Beth, B. F., Elizabeth, O. B., Joseph, B. K., and Mylonakis, E. (2010) Methods for using *Galleria mellonella* as a model host to study fungal pathogenesis. *Virulence*, **1**, 475–482.
- Bouchier-Hayes, L. (2002) CARD games in apoptosis and immunity. *EMBO Rep.*, **3**, 616–621.
- Brennan, M. (2002) Correlation between virulence of *Candida albicans* mutants in mice and *Galleria mellonella* larvae. *FEMS Immunol. Med. Microbiol.*, **34**, 153–157.
- Chen, H. and Fink, G. R. (2006) Feedback control of morphogenesis in fungi by aromatic alcohols. *Genes Dev.*, **20**, 1150–1161.
- Fallon, A. and Sun, D. (2001) Exploration of mosquito immunity using cells in culture. *Insect. Biochem. Mol. Biol.*, **31**, 263–278.
- Fuchs, B. B., Eby, J., Nobile, C. J., El Khoury, J. B., Mitchell, A. P. et al. (2012) Role of filamentation in *Galleria mellonella* killing by *Candida albicans*. *Microbes. Infect.*, **12**, 488–496.
- Grubb, S. E., Murdoch, C., Sudbery, P. E., Saville, S. P., Lopez-Ribot, J. L. et al. (2008) *Candida albicans*-endothelial cell interactions: a key step in the pathogenesis of systemic Candidiasis. *Infect. Immun.*, **76**, 4370–4377.
- Hastings, J. W. and Neilson, K. H. (1977) Bacterial bioluminescence. *Annu. Rev. Microbiol.*, **31**, 549–595.
- Hoffmann, J. A., Kafatos, F. C., Janeway, C. A., and Ezekowitz, R. A. (1999) Phylogenetic perspectives in innate immunity. *Science*, **284**, 1313–1318.
- Hornby, J. M., Jacobitz-Kizzier, S. M., McNeel, D. J., Jensen, E. C., Treves, D. S. et al. (2004) Inoculum size effect in dimorphic fungi: Extracellular control of yeast-mycelium dimorphism in *Ceratomyces ulmi*. *Appl. Environ. Microbiol.*, **70**, 1356–1359.
- Jacobsen, I. (2014) *Galleria mellonella* as a model host to study virulence of *Candida*. *Virulence*, **5**, 237–239.
- Li, D. D., Deng, L., Hu, G. H., Zhao, L. X., Hu, D. D. et al. (2013) Using *Galleria mellonella*-*Candida albicans* infection model to evaluate antifungal agents. *Biol. Pharmaceut. Bull.*, **36**, 1482–1487.
- Lindsay, A. K., Deveau, A., Piispanen, A. E., and Hogan, D. A. (2012) Farnesol and cyclic AMP signaling effects on the hypha-to-yeast transition in *Candida albicans*. *Eukaryot Cell*, **11**, 1219–1225.
- Lo, H. J., Köhler, J. R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A. et al. (1997) Nonfilamentous *C. albicans* mutants are avirulent. *Cell*, **90**, 939–949.
- Mayer, F. L., Wilson, D., and Hube, B. (2013) *Candida albicans* pathogenicity mechanisms. *Virulence*, **4**, 119–128.
- McIlwain, D., Berger, T., and Mak, T. (2013) Caspase functions in cell death and disease. *Cold Spring Harb. Perspect. Biol.*, **5**, a008656–a008656.
- McMillan, S., Verner-Jeffreys, D., Weeks, J., Austin, B., and Desbois, A. (2015) Larva of the greater wax moth, *Galleria mellonella*, is a suitable alternative host for studying virulence of fish pathogenic *Vibrio anguillarum*. *BMC Microbiol.*, **15**, 127.
- Mesa-Arango, A. C., Forastiero, A., Bernal-Martínez, L., Cuenca-Estrella, M., Mellado, E. et al. (2013) The non-mammalian host *Galleria mellonella* can be used to study the virulence of the fungal pathogen *Candida tropicalis* and the efficacy of antifungal drugs during infection by this pathogenic yeast. *Med. Mycol.*, **51**, 461–472.
- Mitchell, A. (1998) Dimorphism and virulence in *Candida albicans*. *Cur. Op. Microbiol.*, **1**, 687–692.
- Mowlds, P., Barron, A., and Kavanagh, K. (2008) Physical stress primes the immune response of *Galleria mellonella* larvae to infection by *Candida albicans*. *Microbes Infect.*, **10**, 628–634.
- Neilson, K. H. and Hastings, J. W. (1979) Bacterial bioluminescence: its control and ecological significance. *Microbiol. Rev.*, **43**, 496–518.
- Nickerson, K. W., Atkin, A. L., and Hornby, J. M. (2006) Quorum sensing in dimorphic fungi: farnesol and beyond. *Appl. Environ. Microbiol.*, **72**, 3805–3813.
- Odds, F. (1996) Epidemiological shifts in opportunistic and nosocomial *Candida* infections: mycological aspects. *Int. J. Antimicrob. Agents*, **6**, 141–144.
- Pappas, P. G., Kauffman, C. A., Andes, D., Benjamin, D. K., Jr., Calandra, T. F. et al. (2009) Clinical practice guidelines for the management of Candidiasis: 2009 update by the Infectious Diseases Society of America. *Clin. Infect. Dis.*, **48**, 503–535.
- Scorzoni, L., de Lucas, M. P., Mesa-Arango, A. C., Fusco-Almeida, A. M., Lozano, E. et al. (2013) Antifungal efficacy during *Candida krusei* infection in non-conventional models correlates with the yeast *in vitro* susceptibility profile. *PLoS ONE*, **8**, e60047.
- Shen, J., Cowen, L., Griffin, A., Chan, L., and Kohler, J. (2008) The *Candida albicans* pescadillo homolog is required for normal hypha-to-yeast morphogenesis and yeast proliferation. *Proc. Natl. Acad. Sci. USA*, **105**, 20918–20923.
- Tsai, C., Loh, J., and Proft, T. (2016) *Galleria mellonella* infection models for the study of bacterial diseases and for antimicrobial drug testing. *Virulence*, **7**, 214–229.
- Vogel, H., Altincicek, B., Glockner, G., and Vilcinskis, A. (2011) A comprehensive transcriptome and immunogene repertoire of the lepidopteran model host *Galleria mellonella*. *BMC Genomics*, **12**, 308.
- Wang, L. and Lin, X. (2012) Morphogenesis in fungal pathogenicity: Shape, size, and surface. *PLoS Pathogens*, **8**, e1003027.
- Wenzel, R. (1995) Nosocomial Candidemia: Risk factors and attributable mortality. *Clin. Infect. Dis.*, **20**, 1531–1534.
- Wojda, I. (2016) Immunity of the greater wax moth *Galleria mellonella*. *Insect. Sci.*, **24**, 342–357.
- Wojda, I., Kowalski, P., and Jakubowicz, T. (2009) Humoral immune response of *Galleria mellonella* larvae after infection by *Beauveria bassiana* under optimal and heat-shock conditions. *J. Insect. Physiol.*, **55**, 525–531.
- Wongsuk, T., Pumeesat, P., and Luplertlop, N. (2016) Fungal quorum sensing molecules: Role in fungal morphogenesis and pathogenicity. *J. Basic Microbiol.*, **56**, 440–447.