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Inhibition of Yeast-to-Filamentous Growth Transitions in *Candida albicans* by a Small Molecule Inducer of Mammalian Apoptosis

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Abstract

The opportunistic fungal pathogen of humans *Candida albicans* is able to grow in different morphological forms such as round or oval yeasts and filamentous hyphae and pseudohyphae. Morphogenesis, the ability to switch between the yeast and filamentous growth forms, is important for adapting to new microenvironments in the human host and for pathogenesis. The molecular pathways governing morphogenesis are complex and incompletely understood. Previously, we identified several small organic molecules that specifically inhibit the initiation of hyphal growth in *C. albicans* without affecting cell viability or budded growth. One molecule from that screen is known to induce apoptosis in mammalian cells. In this study, we have screened additional inducers of mammalian apoptosis and identified BH3I-1, as well as several structural derivatives of BH3I-1, that act as specific inhibitors of morphogenesis under a variety of environmental conditions. Chemical epistasis experiments suggest that BH3I-1 acts downstream of the hypha-specific gene regulators Rfg1, Nrg1 and Ume6.

Keywords

Candida albicans; hyphal growth; morphogenesis; small molecule inhibitors

INTRODUCTION

Candida albicans is a commensal organism of healthy humans that colonizes the mucosal linings of the oral cavity, esophagus, gastrointestinal tract and urogenital system. *C. albicans* is also a major opportunistic pathogen of immunocompromised individuals, including AIDS patients, cancer patients undergoing chemotherapy and individuals receiving tissue transplants. A remarkable aspect of *C. albicans* biology is its ability to grow in different morphological forms such as budding yeast (round or oval cells), and a range of filamentous forms that include true hyphae (filamentous cells without constrictions at the sites of septation) as well as pseudohyphae (chains of elongated cells with visible constrictions at the sites of septation) (Sudberry *et al.* 2004). *C. albicans* can change between yeast-like cells and the various filamentous forms, and the ability to switch growth forms is thought to be

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important for adapting to new environmental niches within its human host, as well for pathogenicity (Lo *et al.* 1997; Gow *et al.* 2002; Saville *et al.* 2003).

The switch from yeast to hyphal growth *in vitro* occurs in response to a variety of environmental stimuli (e.g., serum, 37°C temperature, neutral pH, nutrient limitation, etc.). The response to these signals involves numerous interacting signal transduction pathways, including Ras-MAP Kinase and cAMP-dependent Protein Kinase pathways, and a complex network of transcriptional repressors and activators (e.g., Tup1, Nrg1 and Ume6), ultimately leading to the stable expression of hypha-specific genes and hyphal growth (Sudbery 2011). Despite the fact that many of the components involved in the regulation and execution of hyphal growth have been identified, there is not yet a complete picture of the molecular mechanisms underlying this process.

Since *C. albicans* is an obligate diploid without a facile sexual cycle, classical genetic screens to dissect complex molecular processes are not possible in this organism. Chemical genetics (i.e., the use of bioactive small molecules to inhibit specific cellular proteins) has the potential to circumvent this inherent difficulty and greatly advance research on this important human pathogen. In order develop a set of tools for investigating morphogenesis, we have undertaken large-scale chemical screens to identify small molecules that inhibit the yeast-to-hypha transition without affecting yeast growth. In one such screen, we discovered that a small molecule inducer of mammalian apoptosis also specifically inhibits the yeast-to-hypha transition in *C. albicans* (Toenjes *et al.* 2005). Here we have investigated additional inducers of mammalian apoptosis and have found that one molecule, called BH3I-1, effectively blocks yeast to hyphal and yeast to pseudohyphal growth transitions under a variety of environmental and genetic conditions without inhibiting yeast growth or overall cell viability. We have also screened 120 structural derivatives of BH3I-1 for the ability to inhibit morphogenesis and have found several compounds with activity levels similar to that of BH3I-1. BH3I-1 adds to a growing set of small molecules with anti-morphogenic activity (Shareck and Belhumeur 2011; Tsang *et al.* 2012; Wang *et al.* 2012; Fazly *et al.* 2013; VEDIYAPPAN *et al.* 2013; Sun *et al.* 2015). Identification of the molecular target of BH3I-1, as well as the targets of other known small molecule inhibitors of filamentous growth, may be a fruitful approach to discovering novel proteins critical to *Candida* morphogenesis.

MATERIALS AND METHODS

Candida strains and growth conditions

C. albicans SC5314 or BWP17 were used as the “wild type”. To create the pHWP1-Green Fluorescent Protein (GFP) strains, BWP17 was transformed with pHWP1-GFP (Staab *et al.* 2003) and conditional mutants were transformed with pHWP1-GFP-HIS1 (pHWP1-GFP with the *C. albicans* *HIS1* gene inserted at the *XbaI* restriction site). For standard yeast growth conditions, *C. albicans* strains were grown in YNB (Yeast Nitrogen Base, Ammonium Sulfate, Succinic Acid, 2% glucose, supplemented with appropriate amino acids and, when necessary, uridine) at 30°C or YPD (2% Yeast Extract, 1% peptone and 2% glucose). Spider medium consisted of 1% Nutrient broth, 1% mannitol and 2% K₂HPO₄ supplemented with uridine, arginine and histidine (Liu *et al.* 1994). Lee’s medium consisted of 0.5% (NH₄)₂SO₄, 0.02% MgSO₄, 0.5% NaCl, 1.25% glucose, 0.25% K₂HPO₄

supplemented with uridine, arginine, histidine, alanine, leucine, lysine, methionine, ornithine, phenylalanine, proline, threonine and biotin (Lee *et al.* 1975).

Microplate-based morphological assay

Assays for the inhibition of morphogenesis were carried out in 384-well optical microtiter plates (Toenjes *et al.* 2005). Briefly, cells were first grown overnight in YNB medium at 30°C to promote yeast-form growth, diluted 1:1000 into fresh YNB medium, and then diluted 1:20 into 100 µl of a hypha inducing medium (Spider, Lee's or YPD plus 10% serum). This dilution protocol results in approximately 100 to 200 cells per well. Typically, germ tubes form in nearly 90% of wild type cells within four hours of transfer to a hyphal inducing medium without inhibitory molecule. To test for the ability of small molecules to inhibit morphogenesis, molecules were added to the hypha-inducing media prior to the addition of the yeast-form cells. The “no molecule” control wells always included Dimethyl Sulfoxide (DMSO) at a final concentration of 2%. Plates were incubated at 37°C. Germ tube formation and hyphal elongation were monitored microscopically over the course of 4 hours.

To test for inhibition of morphogenesis in conditional mutants, cells were first grown overnight in YNB without cysteine and methionine (the *MET3* promoter is active under this growth condition), diluted 1:1000 into fresh YNB medium, then diluted 1:20 into 100 µl YNB medium supplemented with 2.5 mM cysteine and 2.5 mM methionine (the *MET3* promoter is repressed under this growth condition). Plates were incubated for 24 hours at 30°C. To test for the ability of small molecules to inhibit morphogenesis, molecules were added to appropriate wells prior to the addition of cells. The “no molecule” control wells always included Dimethyl Sulfoxide (DMSO) at a final concentration equivalent to that of wells with the minimum inhibitory concentration of molecules.

For quantifying hyphal inhibition, cells were scored as hyphal (if they had a long germ tube with no obvious constrictions along the length of the germ tube) or non-hyphal (if they were round or oval, or had obvious constrictions between attached cells). A group of cells that appeared to be the result of division from an original yeast cell was counted as one non-hyphal cell. Typically 50 to 100 cells were counted for each treatment. The percentage of hyphal cells was calculated by dividing the number of hyphal cells by the total number of cells (both hyphal and non-hyphal). A Student's *t*-test was used for statistical comparison between each treatment and the control condition.

“Spot Assay” for cell viability

To test the overall viability of *C. albicans* in BH3I-1, an overnight culture of yeast-form cells was diluted to an OD₆₀₀ of 1.0 and incubated with 0, 25, 50, 100 150, 200 and 300 µM BH3I-1 in YNB medium or Spider medium for 24 h at 30°C. Following the 24 hour incubation period, 5 µl aliquots of undiluted, 10-fold diluted, 100-fold diluted and 1000-fold diluted cells from each BH3I-1 concentration were spotted onto YNB plates (without BH3I-1) and incubated at 30°C for 48 hours.

Construction of conditional *C. albicans* mutants

The *tup1*, *rfg1* and *nrg1* conditional strains were constructed by first replacing one allele with the nourseothricin resistance gene *SAT1*, followed by placement of the second allele under the control of the repressible *MET3* promoter (Care *et al.* 1999; Reuss *et al.* 2004). Briefly, approximately 1000 base pair (bp) PCR fragments from upstream and downstream of the open reading frame (ORF) of the relevant genes were inserted on either side of the *SAT1/FLP* construct of pSFS2 to make the pSFS2 *tup1*, *rfg1* and *nrg1* knockout plasmids. *C. albicans* strain BWP17 was transformed with the pSFS2 knockout plasmids using previously described methods to disrupt one allele of the relevant gene. The remaining intact ORF was placed under the control of the *MET3* promoter by inserting the first ~ 1000 bp of the relevant gene between the *Bam*HI and *Pst*I sites of pCaDis. pCaDis was then digested with an appropriate restriction endonuclease to linearize the plasmid and transformed into the single allele knockout strain to create the conditional strain. To create the *cdc4* conditional strain both copies of *CDC4* were disrupted using pSFS2-CDC4 and *CDC4* was subsequently reintroduced under the control of the *MET3* promoter at the *RPS10* locus in CAI4. After deletion of the first allele the *SAT1* nourseothricin resistance gene was “popped out” by incubation at 30°C for 4 hours in YPS (Yeast Extract, Peptone, 2% Sucrose) to activate expression of the flipase. Popouts were selected for by plating on YPD + 25 mM nourseothricin. Small colonies were picked and plated on YPD as well as YPD + 200 mM nourseothricin to test for nourseothricin sensitivity. Nourseothricin sensitive strains were again transformed with pSFS2CDC4 to knock out the second *CDC4* allele followed by “pop out” of *SAT1*. Strains displaying a *cdc4* phenotype were transformed with pCaExpCDC4 to create the *cdc4::FLP/cdc4::FLP MET3CDC4* strain. Construction of the conditional *C. albicans grr1* strain has been described (Butler *et al.* 2006). All knockouts and insertions were confirmed via Southern blot. All primer sequences and plasmids constructed for this study are available upon request.

Microscopy

Brightfield and fluorescence microscopy was performed on a Nikon TE200 inverted microscope. GFP fluorescence was visualized with a FITC HYQ filter cube. Digital images were captured through either a 10X objective (Figures 2A, 3A, 4, 5 and 6) or a 20X objective (Figure 3B) with a QImaging QICAM 12bit Mono Fast 1394 Cooled camera and QCapture software, and images were minimally processed with Adobe Photoshop software.

Small organic molecules

BH3I-1, BH3I-1', BH3I-1'', BH3I-2, antimycin A, chelerythrine and sanguinarine were purchased from Sigma and EM20–25 was purchased from EMD Millipore. Molecules were resuspended as 10 mM stocks in either DMSO or ethanol according to manufacturers recommendations and stored at –20°C. BH3I-1 derivatives were identified from the 2D & 3D analogs of BH3I-1 at Hit2Lead.com (ChemBridge Corporation) as well as a related compounds search at PubChem Compound (<http://www.ncbi.nlm.nih.gov/pccompound>).

RESULTS

The mammalian apoptotic inducer BH3I-1 inhibits the transition to hyphal growth in *C. albicans*

Previously we discovered 16 small molecules from the BIOMOL-ICCB Known Bioactives collection (Harvard Medical School, Boston, MA, USA) that inhibit starvation-induced hyphal growth without affecting overall cell viability or budded growth of *C. albicans* (Toenjes *et al.* 2005). These molecules are structurally unrelated and affect a variety of targets/pathways in non-fungal cells. One molecule from this set, called HA14-1, was originally identified as an inducer of mammalian apoptosis (Wang *et al.* 2000). For the present study we investigated additional inducers of mammalian apoptosis for the ability to inhibit hyphal development. These small molecules are structurally diverse and inhibit different targets in the apoptosis pathways (see Figure 1 for names and structures).

For the initial screen, 100–200 cells from an overnight culture of wild type yeast-form cells were inoculated into triplicate wells of a microtiter plate containing liquid hypha inducing media (Spider medium, YPD + 10% serum and Lee's medium) at 37°C either with no small molecule or with various concentrations of small molecules ranging from 1 to 300 µM, depending on solubility. Germ tube formation and hyphal extension were monitored microscopically for 4 hours after inoculation. Nine molecules had no inhibitory effect on hyphal development up to the solubility limit of the molecules (BH3I-2, YC-137, Bcl-2 Inh, EM20–25, BH3I-1", Dolastatin 15, C-ATR, Antimycin A, 17-ADMG). Two molecules (Chelerythrine and Sangunarine) were initially scored as cytotoxic at the lowest concentration tested (1 µM), in that no cell division was apparent at 4 hours. Subsequent testing of Chelerythrine and Sangunarine at sub-micromolar concentrations for 24 hours post induction supported the conclusion of cytotoxicity (data not shown). Only one molecule, BH3I-1, appeared to specifically inhibit the yeast-to-hypha transition in all three media while apparently still allowing yeast-form growth.

To confirm the initial results with BH3I-1, we re-tested this molecule at a range of concentrations (25, 50, 75 and 100 µM) in Spider medium (Figure 2A). In this experiment, BH3I-1 significantly inhibited hyphal development above 75 µM (Figure 2A and Figure S1B). Above 25 µM BH3I-1, the non-hyphal cells were moderately elongated and seemed to remain attached after division, suggesting that BH3I-1 may not fully lock cells into a yeast growth program. However, cells grown in YNB (a yeast growth medium) at the same concentrations of BH3I-1 showed similar cell morphologies (Supplemental Figure S1A). As a molecular reporter of hyphal growth, we also monitored expression of the Green Fluorescent Protein (GFP) under the control of the hypha-specific *HWPI* promoter. In Spider medium without BH3I-1, the *HWPI-GFP* reporter gene was strongly expressed, whereas at 100 µM, BH3I-1 prevented *HWPI-GFP* expression for up to 24 hours post induction (Figure 2B). BH3I-1 does not inhibit hyphal development through general cell toxicity. "Spot assays" carried out in YNB and Spider media showed that there is no loss of viability up to 150 µM BH3I-1 (Figure 2C and Supplemental Figure S1C). Thus, at an appropriate concentration, BH3I-1 can act as a specific inhibitor of hyphal development without affecting the growth of non-hyphal cells.

BH3I-1 inhibits filamentous growth in *tup1*, *nrg1*, *rfg1* and *cdc4* mutants

The expression of hypha-specific genes (HSGs) is negatively regulated by the transcriptional repressor complexes Nrg1/Tup1 and Rfg1/Tup1 (Sudbery 2011). A current model for hyphal development postulates that the initiation of hyphal growth involves displacement of the Nrg1/Tup1 repressor complex from HSG promoters (Lu *et al.* 2011; Lu *et al.* 2014). Subsequent, chromatin remodeling at HSG promoters prevents further binding of the repressor complex but allows binding of the transcriptional activator Ume6 (and most likely other transcriptional activators as well), thus stabilizing HSG expression and maintaining hyphal growth. Consistent with the foregoing, deletion of *TUP1*, *NRG1* or *RFG1*, or over-expression of *UME6* results in constitutive filamentous growth (Braun and Johnson 1997; Kadosh and Johnson 2001; Khalaf and Zitomer 2001; Murad *et al.* 2001; Banerjee *et al.* 2008).

We wanted to investigate whether BH3I-1 can block the initiation of filamentous growth when the negatively acting Nrg1/Tup1/Rfg1 pathways are inactivated. For these experiments we constructed a series of “conditional” mutant strains, wherein one allele of the gene of interest (either *TUP1*, *RFG1* or *NRG1*) was knocked out and the remaining wild type allele was placed under the control of the repressible *MET3* promoter. The *MET3* promoter is induced in media lacking methionine and cysteine, but is rapidly repressed in the presence of these amino acids (Care *et al.* 1999). When the conditional mutant strains were grown without met/cys (inducing the *MET3* promoter), round or oval yeast-like cells were exclusively observed. In contrast, when mutant cells were transferred to medium with met/cys (repressing the *MET3* promoter), greater than 95% of the culture showed a highly filamentous morphology by 24 hours after transfer (Figure 3). Repression of the *MET3* promoter was then carried out in the presence of 50, 100 and 200 μ M BH3I-1. BH3I-1 clearly inhibited the formation of filamentous cells in the *nrg1* and *rfg1* mutants at 100 μ M, with no evidence of cytotoxicity. BH3I-1 also completely blocked expression of *HWPI-GFP* in both the *nrg1* and *rfg1* strains in met/cys. The results with *tup1* were less clear-cut. BH3I-1 appeared to only partially inhibit the formation of filaments in this mutant, in that the cells appeared more pseudohyphal-like rather than yeast-like in the presence of the molecule (Figure 3).

We carried out a similar experiment with a conditional *CDC4* knockout. The *CDC4* gene encodes the recognition subunit of a ubiquitin ligase that targets Ume6 for degradation in yeast-form cells. Deletion of *CDC4* results in constitutive filamentous growth, primarily due to the aberrant stabilization of Ume6 (Mendelsohn *et al.* 2017). As with repression of *NRG1* and *RFG1*, 100 μ M BH3I-1 was also able to nearly completely inhibit morphogenesis when *CDC4* expression was repressed (Fig 3C).

BH3I-1 inhibits the switch to pseudohyphal growth

We also tested whether BH3I-1 has the ability to block pseudohyphal morphogenesis. In previous work, we found that deletion of *GRR1* results in constitutive pseudohyphal growth (Butler *et al.* 2006; Li *et al.* 2006). *GRR1*, like *CDC4*, encodes a ubiquitin ligase subunit that selectively targets a number of proteins for degradation. As with the foregoing experiments, we used a “conditional” *MET3-GRR1* strain to induce pseudohyphal morphogenesis. As

with the hyphae-inducing mutants, BH3I-1 blocked the transition to pseudohyphal growth but at a lower concentration (Figure 4). This observation implies that BH3I-1 acts on a target that is fundamental to all forms of filamentous growth.

Identification of bioactive derivatives of BH3I-1

In order to identify inhibitors of morphogenesis with greater potency than BH3I-1 and to aid in target identification studies, we used the ChemBridge Hit2Lead and PubChem databases to select 120 small molecules that share a core structure (the benzylidene group attached to a thiazolidine ring) with BH3I-1. These BH3I-1 derivatives were tested for the ability to inhibit hyphal morphogenesis in wild type *C. albicans* at concentrations ranging from 1 μ M to 300 μ M. Interestingly, only derivatives with alterations of substituents on the aromatic ring showed increased inhibitory activity in our assay. For example, changing either the position of the bromine or adding two chlorines in place of the bromine lowered the concentration for 90% inhibition from 100 μ M (for BH3I-1) to 50 μ M (see Figure 5 for an example and supplemental figure S2 for two additional examples). Changes in other parts of the molecule either completely abolished activity (i.e., the molecule did not inhibit hyphal morphogenesis at 300 μ M) or did not affect activity (i.e., the molecule inhibited hyphal morphogenesis at the same concentration as BH3I-1). Continued efforts to identify bioactive derivatives of BH3I-1 will focus on molecules with different substituents on the aromatic ring.

DISCUSSION

Small organic molecules have the potential to be important tools for dissecting complex cellular processes, especially in organisms without a classical genetic system such as *C. albicans*. In this report, we present evidence that the small molecule BH3I-1 inhibits yeast-to-filamentous growth transitions under a variety of environmental and genetic conditions *in vitro*. The molecule does not exert a negative effect on the growth of non-filamentous cells (at least at the concentrations needed to inhibit filamentous growth), thus BH3I-1 likely acts on a target specific to the initiation and/or continuation of filamentous growth.

The mechanism-of-action of BH3I-1 in mammalian cells is well established. BH3I-1 was originally identified as a ligand of the Bcl-X_L protein, an anti-apoptotic member of the Bcl-2 family of proteins (Degterev *et al.* 2001). Through binding to Bcl-X_L, BH3I-1 induces apoptosis in mammalian cells by disrupting the antagonistic interaction between Bcl-XL and pro-apoptotic members of the Bcl-2 family. On this basis, it is tempting to speculate that BH3I-1 interferes with filamentation in *C. albicans* through a similar apoptotic pathway. However, bioinformatics analyses have indicated that fungal genomes do not encode any obvious Bcl-2 family orthologs (Madeo *et al.* 2002; Fedorova *et al.* 2005; our unpublished data). Thus we suggest that BH3I-1 acts by a completely novel mechanism in fungi.

While the chemical epistasis experiments reported here do not establish a mechanism-of-action for BH3I-1, they do imply that BH3I-1 acts on a target downstream of the HSG repressor complexes involving Rfg1 and Nrg1, and the transcriptional activator Ume6. Given our observation that expression of the hypha-specific promoter *HWP1* is effectively suppressed by BH3I-1 under hypha-inducing environmental and genetic conditions, it may

be that BH3I-1 directly interferes with the assembly of a competent transcription complex on HSG promoters. Ultimately, identification of the target of BH3I-1 will reveal a critical, and perhaps novel, molecular component of filamentous growth in *C. albicans*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.
Structures of small molecules used in this study.

Figure 2.

(A) Inhibition of the yeast-to-hyphal growth transition in *C. albicans* at 0, 25, 50, 75 and 100 μM BH3I-1. For the experiment shown, induction was carried out with Spider medium. (B) Expression of *HWPI-GFP* reporter gene after 24 hours of incubation in Spider medium with 100 μM BH3I-1. (C) Spot Assay. Viability of *C. albicans* after 24 hours of incubation in YNB with 0, 25, 50, 75, 100, 150, 200 and 300 μM BH3I-1. Following the 24 hour incubation in YNB, 5 μl aliquots of 10-fold serially diluted cells from each BH3I-1 concentration were spotted to YPD plates (without BH3I-1) and incubated for an additional 48 hours.

Figure 3.

(A) BH3I-1 inhibition of filamentous growth in the *MET3-TUP1*, *MET3-NRG1* and *MET3-RFG1* strains after 24 hours. The third and fifth columns are fluorescence images showing *HWP1-GFP* expression in these strains. Exposure time and processing for all fluorescence images were identical. (B) BH3I-1 inhibition of filamentous growth in the *MET3-CDC4* strain. For both (A) and (B), “Expressed” indicates growth conditions under which the *MET3* promoter is active and “Repressed” indicates growth conditions under which the *MET3* promoter is not active. “No Molecule” is the DMSO control and “BH3I-1” is the molecule administered at 100 μ M.

Figure 4.

Inhibition of the yeast-to-pseudohyphal growth transition in the *MET3-GRR1* strain by BH3I-1. “Expressed” indicates growth conditions under which the *MET3* promoter is active and “Repressed” indicates growth conditions under which the *MET3* promoter is not active. “No Molecule” is the DMSO control and “BH3I-1” is the molecule administered at 50 μ M.

Figure 5.
Inhibition of the *C. albicans* yeast-to-hyphal growth transition by a BH3I-1 derivative.