Journal of Medical Microbiology (2009), **58**, 779–790 **DOI** 10.1099/jmm.0.006841-0

Inhibitors of cellular signalling are cytotoxic or block the budded-to-hyphal transition in the pathogenic yeast *Candida albicans*

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Received 23 September 2008
Accepted 10 February 2009

The pathogenic yeast *Candida albicans* can grow in multiple morphological states including budded, pseudohyphal and true hyphal forms. The ability to interconvert between budded and hyphal forms, herein termed the budded-to-hyphal transition (BHT), is important for *C. albicans* virulence, and is regulated by multiple environmental and cellular signals. To identify small-molecule inhibitors of known cellular processes that can also block the BHT, a microplate-based morphological assay was used to screen the BIOMOL–Institute of Chemistry and Cell Biology (ICCB) Known Bioactives collection from the ICCB-Longwood Screening Facility (Harvard Medical School, Boston, MA, USA). Of 480 molecules tested, 53 were cytotoxic to *C. albicans* and 16 were able to block the BHT without inhibiting budded growth. These 16 BHT inhibitors affected protein kinases, protein phosphatases, Ras signalling pathways, G protein-coupled receptors, calcium homeostasis, nitric oxide and guanylate cyclase signalling, and apoptosis in mammalian cells. Several of these molecules were also able to inhibit filamentous growth in other *Candida* species, as well as the pathogenic filamentous fungus *Aspergillus fumigatus*, suggesting a broad fungal host range for these inhibitory molecules. Results from secondary assays, including hyphal-specific transcription and septin localization analysis, were consistent with the inhibitors affecting known BHT signalling pathways in *C. albicans*. Therefore, these molecules will not only be invaluable in deciphering the signalling pathways regulating the BHT, but also may serve as starting points for potential new antifungal therapeutics.

INTRODUCTION

Candida albicans is a major opportunistic pathogen of immunocompromised hosts (Schmidt-Westhausen *et al.*, 1991; Warnock, 2007), as well as a leading cause of nosocomial bloodstream infections, especially in patients with indwelling medical devices (Kojic & Darouiche, 2004; Lynch & Robertson, 2008). *C. albicans* can grow as either budded (yeast-like) or filamentous cells, the latter comprising pseudohyphae and true hyphae (Sudbery *et al.*, 2004). Both budded and hyphal cells are found at sites of infection, and the ability to undergo the budded-to-hyphal transition (BHT) is important for virulence (Saville *et al.*, 2003). *C. albicans* mutants that have defects in the BHT have a reduced ability to become internalized and to cause endothelial cell injury *in vitro* (Phan *et al.*, 2000), and small-molecule inhibitors of the BHT can protect endothelial cells from *C. albicans*-induced cell damage (Toenjes *et al.*, 2005). These data are indicative that the BHT can modulate the ability of *C. albicans* to cause endothelial cell injury and suggest that the BHT is critical for systemic candidiasis.

The BHT occurs in response to a variety of environmental signals, including temperature above 35 °C, pH above 6.5, nutrient starvation and growth in serum (Ernst, 2000). Therefore, it is not surprising that multiple signalling pathways regulate the BHT (Biswas *et al.*, 2007; Brown, 2002). Genetic analysis of these signalling pathways has been hampered by the inability to isolate recessive loss-of-function mutants in the diploid *C. albicans*. For this reason, a 'forward genetic' approach using small-molecule inhibitors to study the BHT was initiated (Toenjes *et al.*, 2005).

Abbreviations: BHT, budded-to-hyphal transition; ETYA, 5,8,11,14-eicosatetraynoic acid; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; ICCB, Institute of Chemistry and Cell Biology; NO, nitric oxide; PKA, protein kinase A.

A table of the structures of the BHT inhibitors is available as supplementary material with the online version of this paper.

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The use of small organic molecules in deciphering complex biological processes has been very informative (Lokey, 2003; Nehil *et al.*, 2007; Ward *et al.*, 2002). Previously, we developed a microplate-based morphological assay that identified seven novel small molecules that inhibited the BHT without affecting budded growth (Toenjes *et al.*, 2005). To augment this forward genetic approach, the BIOMOL–Institute of Chemistry and Cell Biology (ICCB) Known Bioactives collection, which contains 480 molecules that inhibit known cellular targets or processes, was screened for cytotoxic molecules and BHT inhibitors. We identified 53 molecules that were cytotoxic to *C. albicans* and 16 molecules that inhibited the BHT without affecting budded growth. Several of these molecules also inhibited filamentous growth in the pathogenic filamentous fungus *Aspergillus fumigatus*. This raises the possibility that these molecules or their bioactive derivatives may be good starting points for the development of new antifungal therapeutics.

METHODS

C. albicans strains and media. Protocols for the growth and maintenance of *C. albicans* strains have been described previously (Toenjes *et al.*, 2005). Growth in nutrient-limiting Spider medium (Liu *et al.*, 1994) was used to induce hyphal growth at 37 °C, as described previously (Toenjes *et al.*, 2005). The following *C. albicans* strains were used in this study: SC5314 (wild-type clinical isolate); YAW2 (*pADH-CDC10-GFP ura3*::λ*imm434/ura3*::λ*imm434 his1*::*hisG/his1*::*hisG arg4*::*hisG/arg4*::*hisG*, kindly provided by James Konopka, Stony Brook University, Stony Brook, NY, USA (Warenda & Konopka, 2002; Warenda *et al.*, 2003); and KTCa1 (*URA3-pHWP1-GFP ura3*::*limm434/ura3*::*limm434*; Toenjes *et al.*, 2005), which has the green fluorescent protein (GFP) encoding gene expressed under the control of the hyphal-induced *HWP1* promoter (*HWP1* pr–GFP). Induction of *HWP1* pr–GFP expression was observed upon growth in Spider medium at 37 °C.

Microplate-based morphological assay. The microplate-based morphological assay utilized in these studies has been described previously (Toenjes *et al.*, 2005). In the initial screen, *C. albicans* SC5314 cells from a single colony were grown in YNB medium (0.67 % yeast nitrogen base, 2 % glucose and required amino acids, pH 7) overnight at 30 °C with shaking. The culture was diluted 1 : 25 000 into hyphal-inducing Spider medium and 100 μl was placed into each well of a 384-well microplate containing small molecules from the BIOMOL–ICCB Known Bioactives collection (BIOMOL International, LP, Plymouth Meeting, PA, USA; see http://iccb.med.harvard.edu/screening/compound_libraries/bioactives_biomol_med.htm for a list of the molecules) obtained from the ICCB-Longwood Screening Facility (Harvard Medical School, Boston, MA, USA). Molecules were tested at two concentrations: high concentration was ~130 μM for most compounds or ~13 μM for 'potent' compounds (see http://iccb.med.harvard.edu for more details), whereas medium concentration was ~30 μM for most compounds or ~3 μM for 'potent' compounds. Plates were incubated at 37 °C for 4 h, at which time the cells in each well were fixed by adding glutaraldehyde (electron microscopy grade; Acros Organics/Fisher Scientific) to a final concentration of 5 % (v/v) for light microscopy.

Quantification of BHT inhibition was accomplished by counting the number of individual budded cells versus the number of hyphae in the population. More than 100 cells were counted for each well and all assays were repeated at least twice. Those molecules that showed more than 95 % budded cells after 4 h were examined further. The IC_{100} value for selected BHT inhibitory molecules (i.e. lowest concentration of molecule used at which 100 % budded cells were observed) was determined using serial dilutions from 200 to 1 μM of the individual molecules.

To test for cytotoxicity, *C. albicans* cells were incubated with the molecule in Spider medium for 24 h at 37 °C, after which time the wells were checked visually for turbidity and the cells examined morphologically. If there was no difference between the 4 and 24 h time points, the molecules were deemed either cytotoxic or cytostatic. The BHT inhibitors displayed significant budded growth over the 24 h time period. To verify further that the BHT inhibitors were not cytotoxic, the well contents from the 24 h incubations were resuspended by pipetting and 5 μl plus serial dilutions were incubated on YPD + uridine plates at 37 °C to test for growth.

The reversibility assay was performed as described previously (Toenjes *et al.*, 2005). Briefly, the growth medium + molecule was removed after 4 h of incubation at 37 °C, and the cells were washed once with Spider medium, resuspended in 100 μl of fresh Spider medium without the molecule and incubated for 8 h at 37 °C before fixation.

A. fumigatus conidial germination. *A. fumigatus* clinical isolate strain Af293, from which the *Aspergillus* genomic sequence has been determined (Nierman *et al.*, 2005), was kindly provided by Dr Kieren Marr, University of Washington, Seattle, WA, USA. Approximately 1×10^5 conidia from strain Af293 were incubated in either YNB medium (germination negative control) or Spider medium (germination positive control) with 100 μM selected BHT inhibitor for 8 h at 37 °C and then fixed for microscopic examination. The reversibility of these molecules was tested by incubation of the *A. fumigatus* with the molecules for 8 h followed by removal of the medium, washing with Spider medium and reincubation with fresh Spider medium without the molecules for 8 h. Inhibition of hyphal elongation was tested by incubating *A. fumigatus* conidia in Spider medium without the molecule for 7 h at 37 °C to allow hyphae to form, followed by removal of the medium, replacement with Spider medium containing the molecule and incubation for an additional 5 h at 37 °C.

Microscopy techniques. For light microscopy, *C. albicans* cells in 384-well microplates with an optical plastic bottom (BD Falcon) were routinely viewed on a Nikon TE300 or TE200 microscope with differential interference contrast/Hoffman optics and a 20× objective. Images of each well were obtained either using a SpotRT monochrome camera (TE300) or driven by *In Vivo* software (QED Imaging). For fluorescent microscopy, *C. albicans* cells in 384-well microplates with an optical-glass bottom (BD Falcon) were viewed on a Nikon TE300 microscope using Nikon filter sets. *HWP1* pr–GFP expression was observed using KTCa1 cells in the assay as described previously (Toenjes *et al.*, 2005), using a GFP HYQ filter set and a 20× objective. Localization of the Cdc10p septin protein in YAW2 cells was as described previously (Toenjes *et al.*, 2005), using a Nikon TE200 inverted microscope, a GFP HYQ filter set and a 60× objective.

RESULTS AND DISCUSSION

Current antifungal therapies are limited to drugs such as amphotericins, azoles and echinocandins that inhibit the growth of *C. albicans* cells rather than specific virulence processes. Unfortunately, these drugs can have inhibitory effects on human cells, leading to serious side effects for the

host. Therefore, new approaches to discovering possible antifungal therapeutics that target specific virulence factors and processes are needed. One such approach could be based on the ability of small molecules to inhibit the BHT without affecting the growth of *C. albicans* cells. Data from a number of laboratories have been indicative that the BHT is required for *C. albicans* virulence (Saville *et al.*, 2003).

Identification of cytotoxic molecules

A microplate-based morphogenesis assay (Toenjes *et al.*, 2005) was used to screen the BIOMOL–ICCB Known Bioactives collection, which contains 480 small molecules whose mammalian cellular targets and/or biological activities have been well characterized. The initial assay was for molecules that inhibited *C. albicans* hyphal formation in Spider medium, i.e. only budded cells were observed in the wells. This inhibition could be the result of either cytotoxicity of the molecule or inhibition of the BHT. Initially, two concentrations of molecules were assayed. The medium concentration (~30 μM concentration) screen yielded 21 molecules that inhibited hyphae formation, whereas the high concentration (~130 μM concentration) screen yielded 74 molecules. Subsequent retesting of the high-concentration collection eliminated five molecules from the screen. Cytotoxicity assays of the remaining 69 molecules indicated that 53 molecules were cytotoxic after incubation for 24 h (Table 1), whereas 16 molecules did not inhibit budded growth but did inhibit the BHT (Table 2).

Among the cytotoxic molecules were several molecules that have known antifungal activity, such as tamoxifen (Beggs, 1993), *β*-lapachone (Guiraud *et al.*, 1994), brefeldin A (Arioka *et al.*, 1991), rapamycin (Cruz *et al.*, 2001) and cerulenin (Hoberg *et al.*, 1983). In addition to the protein kinase C inhibitor tamoxifen, 17 other known protein kinase or protein phosphatase inhibitors were identified as cytotoxic (Table 1). The calcineurin phosphatase inhibitor tacrolimus (FK506) was not cytotoxic to *C. albicans*, which has been determined by Steinbach *et al.* (2007), but it did inhibit the BHT (Table 2). Interestingly, ascomycin (FK520), another calcineurin phosphatase inhibitor, along with the phosphatase inhibitor calyculin A were cytotoxic, similar to rapamycin, an FK506-binding protein inhibitor, whereas other calcineurin or phosphatase inhibitors in the collection, such as cyclosporine A, cypermethrin and okadaic acid, had no effect (data not shown). These observations indicated that inhibitors of calcineurin or its regulators could have variable effects on *C. albicans* growth and morphogenesis.

Inhibitors of a variety of other mammalian signalling pathways were cytotoxic to *C. albicans*, including receptor antagonists such as enantio-PAF C16, and molecules that affected calcium and other ion homeostasis such as bepridil and valinomycin. As with the calcineurin inhibitors described above, other molecules in the Known Bioactives collection that inhibited these cellular processes had variable effects on *C. albicans*. Overall, these data reinforce the notion that inhibiting cellular signalling pathways can have a detrimental effect on cell growth, and raise the possibility that one or more of these molecules may have potent antifungal activity that can be exploited in the future.

Of particular interest was the identification of inhibitors of lipooxygenases and cyclooxygenases within the screen. Cyclooxygenases and lipooxygenases convert arachidonic acid to eicosanoids, specifically prostaglandins and leukotrienes, respectively. *C. albicans* cells produce a variety of eicosanoids, which may regulate cell growth, morphogenesis, biofilm formation and virulence (Alem & Douglas, 2005; Noverr *et al.*, 2003). However, no cyclooxygenases or lipooxygenases have been identified within the annotated *C. albicans* genome database. In our assay, addition of arachidonic acid, as well as 40 other eicosanoids or bioactive lipids, such as prostaglandins B1, B2, D2, E1, E2, F2, F1a and I2, and linoleic acid, had no effect on cell viability or the BHT. However, the lipooxygenase inhibitors 5,8,11-eicosatriynoic acid, cinnamyl-3,4-dihydroxy-cyanocinnamate and ebselen were cytotoxic to *C. albicans* (Table 1), whereas the lipooxygenase inhibitor 5,8,11,14-eicosatetraynoic acid (ETYA; IC_{100} 12 μM) inhibited the BHT (Table 2), and the lipooxygenase/cyclooxygenase inhibitors 5(S)-HETE, BW-B 70C, AA-861, NS-398, REV-5901, indomethacin, nimesulide, piroxicam and resveratrol had no effect (data not shown). Indomethacin, nimesulide and piroxicam were previously shown to have modest effects on *C. albicans* hyphal formation and biofilm formation (Alem & Douglas, 2004). Interestingly, aspirin, one of the most commonly used cyclooxygenase inhibitors, has been shown to be cytotoxic and to inhibit *C. albicans* biofilm formation (Alem & Douglas, 2004). As *C. albicans* does not have any recognizable cyclooxygenases or lipooxygenease, it remains to be determined whether ETYA is inhibiting the BHT by inhibiting lipooxygenases and eicosanoid synthesis. Taken together, these data reinforce the notion that eicosanoid synthesis is important for cell viability and regulating the BHT in *C. albicans*, and that different inhibitors of the same cellular enzymes can have differential effects *in vivo*.

Interestingly, the nitric oxide (NO) synthase inhibitors diphenyleneiodonium chloride and thiocitrulline, along with the guanylate cyclase inhibitor LY-83583 and activator furoxan, were cytotoxic to *C. albicans* (Table 1), whereas the guanylate cyclase activator YC-1 (IC_{100} 60 μM) inhibited the BHT (Table 2). NO is an activator of soluble guanylate cyclases in mammalian cells and has been shown to have potent candidacidal activity independent of its guanylate cyclase activation when produced by macrophages within the host (Ullmann *et al.*, 2004). As was seen with other molecules within the collection, certain NO synthase inhibitors (1400W, 3-bromo-7-nitroindazole), guanylate cyclases activators (LY-83583, minoxidil) and cyclic GMP phosphodiesterase inhibitors (MY-5445, MBCQ, 8-bromo-cGMP) had no effect in the assay (data not shown). Surprisingly, there is no recognizable guanylate cyclase or NO synthase encoded in the *C. albicans*

Table 1. Cytotoxic molecules

Unless indicated otherwise, the molecules were tested at high concentrations of ~130 μM against SC5314 cells in Spider medium for 4 h at 37 °C.

Molecule	Mechanism of action	Reference
	Kinases and phosphatases	
5-Iodotubercidin	Adenosine kinase inhibitor, ERK2 inhibitor	Massillon *et al.* (1994)
AG213 (tyrphostin 47)	EGF receptor kinase inhibitor	Yaish *et al.* (1988)
BAY 11-7082*	Inhibits TNF-α-inducible phosphorylation	Pierce *et al.* (1997)
Chelerythrine*	Protein kinase C inhibitor	Herbert *et al.* (1990)
Edelfosine (Et-18-OCH_3)	Protein kinase C inhibitor, Na^+/K^+ ATPase inhibitor	Zheng *et al.* (1990)
AMG-PC (Et-16-OCH_3)	Protein kinase C and diacylglycerol kinase inhibitor	Grosman (1991)
N,*N*-Dimethylsphingosine†	Protein kinase C and sphingosine kinase inhibitor	Yatomi *et al.* (1996)
Geldanamycin	Binds HSP90, tyrosine kinase inhibitor	Whitesell *et al.* (1994)
K252A†	Protein kinase inhibitor, broad spectrum	Kase *et al.* (1987)
ML-7	Myosin light chain kinase inhibitor	Saitoh *et al.* (1987)
ML-9	Myosin light chain kinase inhibitor	Saitoh *et al.* (1987)
Rapamycin	Known antifungal, FK506-binding protein inhibitor	Cruz *et al.* (2001)
Ascomycin (FK520)	FK506-binding protein binder, inhibits calcineurin phosphatase	Wallace *et al.* (1994)
Ro 31-8220	Protein kinase C inhibitor	McKenna & Hanson (1993)
Staurosporine*	Protein kinase inhibitor, broad spectrum	Tamaoki *et al.* (1986)
Tamoxifen*	Known antifungal, protein kinase C inhibitor	Beggs (1993)
Calyculin A†	Protein phosphatase 1 and 2A inhibitor	Ishihara *et al.* (1989)
RK-682*	Protein tyrosine phosphatase inhibitor	Hamaguchi *et al.* (1995)
Anisomycin*	Bacterial translation inhibitor, activates JNK/SAPKs	Zinck *et al.* (1995)
	Receptor antagonists or agonists	
Enantio-PAF C16†	Platelet-activating factor receptor antagonist	Wykle *et al.* (1981)
Mastoparan*	G protein activator	Higashijima *et al.* (1988)
	Ion homeostasis	
Calcimycin (A-23187)	Ca^{2+} ionophore	Gietzen *et al.* (1982)
Bepridil*	Ca^{2+} channel blocker	Zeller & Spinler (1987)
Dichlorobenzamil	Ca^{2+} channel blocker	Xu *et al.* (1999)
FCCP*	Mitochondrial uncoupling agent	Collins *et al.* (2000)
Hinokitiol*	Fe^{2+} chelator, induces apoptosis	Tanaka *et al.* (1999)
Penitrem A	Ca^{2+}-activated K^+ (maxi-K) channel blocker	Knaus *et al.* (1994)
Phenamil	Amiloride-sensitive Na^+ channel inhibitor	Garvin *et al.* (1985)
SK&F-96365	Receptor-mediated Ca^{2+} entry inhibitor	Merritt *et al.* (1990)
Tetrandrine	Ca^{2+} and K^+ channel blocker	Wang & Lemos (1992)
TPEN	Zn^{2+} chelator, induces apoptosis	McCabe *et al.* (1993)
Trifluoperazine*	Calmodulin antagonist, anti-schizophrenic drug	Rao (1987)
Valinomycin*	K^+ ionophore	Pressman (1976)
	NO and guanylate cyclase	
Diphenyleneiodonium chloride	NO synthase inhibitor	Stuehr *et al.* (1991)
Thiocitrulline*	NO synthase inhibitor	Frey *et al.* (1994)
Furoxan	NO donor, guanylate cyclase activator	Medana *et al.* (1994)
LY-83583	Guanylate cyclase inhibitor	Mülsch *et al.* (1989)
	Signalling pathways	
Cerulenin	Known antifungal, protein prenylation inhibitor	Hoberg *et al.* (1983)
Gliotoxin*	Fungal toxin, farnesyltransferase and geranylgeranyltransferase inhibitor	Vigushin *et al.* (2004)
Manumycin A	Farnesyltransferase inhibitor	Hara *et al.* (1993)
U73122*	Phospholipase C activation inhibitor	Smith *et al.* (1990)
Vinpocetine	Phosphodiesterase I inhibitor	Hagiwara *et al.* (1984)
Wiskostatin*	N-WASP inhibitor, inhibits actin filament assembly	Peterson & Mitchison (2002)
Caffeic acid phenethylester	NF-κB inhibitor	Natarajan *et al.* (1996)

Table 1. cont.

Molecule	Mechanism of action	Reference
	Lipooxygenase inhibitors	
5,8,11-Eicosatriynoic acid†	5-, 12- and 15-lipooxygenase inhibitor	Salari *et al.* (1984)
Cinnamyl-3,4-dihydroxycy-anocinnamate	12-Lipooxygenase inhibitor	Cho *et al.* (1991)
Ebselen*	Lipooxygenase and protein kinase C inhibitor	Cotgreave *et al.* (1989)
	Assorted cellular functions	
3,4-Dichloroisocoumarin	Serine protease inhibitor	Powers *et al.* (1989)
β-Lapachone	Known antifungal, inhibits DNA topoisomerase I	Guiraud *et al.* (1994)
Brefeldin A*	Known antifungal, endoplasmic reticulum-to-Golgi transport inhibitor	Klausner *et al.* (1992)
Lycorine	Protein synthesis inhibitor, apoptosis inducer	Vrijsen *et al.* (1986)
Splitomycin	Sir2p histone deacetylase inhibitor	Bedalov *et al.* (2001)
Tunicamycin*	Inhibits *N*-linked glycosylation	Duksin & Mahoney (1982)

EGF, Epidermal growth factor; FCCP, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone; TPEN, *N*,*N*,*N*′,*N*′-tetrakis-(2-pyridylmethyl)-ethylenediamine.

*These molecules were tested at medium concentrations of ~28.5 µM.

†These were high-potency molecules that were tested at concentrations of ~13 µM; cytotoxicity was determined by a lack of cell growth after 24 h.

Table 2. BHT inhibitors

Molecules were tested against SC5314 cells in Spider medium for 4 h at 37 °C. Inhibition of the BHT was determined by a lack of hyphal cells after 4 h incubation.

Molecule (IC_{100})	Mechanism of action	Reference
	Kinases, phosphatases, Ras signalling pathways	
A-3 (60 µM)	PKA inhibitor, broad spectrum	Inagaki *et al.* (1986)
W7 (40 µM)	Protein kinase C inhibitor, calmodulin antagonist	Kawamoto & Hidaka (1984)
Tyrphostin AG1478 (80 µM)	EGF receptor tyrosine kinase inhibitor	Fan *et al.* (1995)
Tyrphostin 9 (80 µM)	EGF and platelet-derived growth factor receptor tyrosine kinase inhibitor	Levitzki & Gilon (1991)
GW 5074 (60 µM)	cRaf-1 kinase inhibitor	Lackey *et al.* (2000)
FK506 (60 µM)	Known antifungal, calcineurin phosphatase inhibitor	Schreiber & Crabtree (1992)
L-744,832 (130 µM)	Farnesyltransferase inhibitor	Barrington *et al.* (1998)
	Receptor antagonists or agonists	
Clozapine (50 µM)	Dopamine GPCR antagonist	Ereshefsky *et al.* (1989)
Fluspirilene (40 µM)	Dopamine receptor antagonist	Sah & Bean (1994)
GW 9662 (130 µM)	Peroxisome proliferator-activated receptor antagonist	Leesnitzer *et al.* (2002)
ETYA (12 µM)	Lipoxygenase, cyclooxygenase inhibitor	Salari *et al.* (1984)
	Ion homeostasis	
CGP-37157 (40 µM)	Mitochondrial Na^{+}/Ca^{2+} exchanger inhibitor	Cox *et al.* (1993)
TMB-8 (40 µM)	Intracellular Ca^{2+} antagonist, inhibits Ca^{2+} release from endoplasmic reticulum	Chiou & Malagodi (1975)
Nigericin (130 µM)	Known weak antifungal, K^{+}/H^{+} ionophore	Vercesi *et al.* (1993)
	NO and guanylate cyclase	
YC-1 (60 µM)	Guanylate cyclase activator	Mülsch *et al.* (1997)
	Apoptosis signalling pathways	
HA14-1 (20 µM)	Bcl-2 inhibitor	Degterev *et al.* (2001)

genome, so the mechanism of action of the BHT inhibitor YC-1 and the other cytotoxic molecules is not obvious.

Clearly, the 53 cytotoxic molecules identified in this study could have powerful roles in antifungal therapeutics, and thus may be excellent starting points for the development of new antifungal drugs. It is important to note that there were many other molecules in the Known Bioactives collection that were predicted to inhibit the same cellular processes as these 53 cytotoxic molecules, yet these molecules had no effect on *C. albicans* growth. This disparity could be partially explained by the inability of some of the inactive molecules to enter the *C. albicans* cell through its cell wall and membrane, although this explanation is unlikely to suffice for all inactive molecules. The fact that different molecules predicted to affect the same cellular process or protein can have differential effects is more likely to be the result of dosage effects or different interactions between the molecules and their cellular targets. Therefore, identifying and characterizing the cellular targets for the BHT inhibitors is a high priority for future studies.

Identification of BHT inhibitors

The 16 BHT inhibitors identified in this study (Table 2; Supplementary Table S1 available with the online journal) were not fungicidal, suggesting that these molecules were not inhibiting essential proteins and processes needed for growth in *C. albicans*. These inhibitors affected a variety of signalling pathway components, including kinases and phosphatases. Among the kinase inhibitors, molecule A-3 (IC_{100} 60 μM) has a broad inhibitory spectrum against protein kinases, but seems to have better efficacy against protein kinase A (PKA) (Inagaki *et al.*, 1986), whereas its structural homologue W7 (IC_{100} 40 μM) is a more effective protein kinase C inhibitor (Kawamoto & Hidaka, 1984; Tanaka *et al.*, 1983). Identification of these kinase inhibitors as BHT inhibitors was particularly encouraging, as the role of PKA in regulating the BHT is well established (Sonneborn *et al.*, 2000). The two tyrphostin molecules AG1478 (IC_{100} 80 μM) and tyrphostin 9 (IC_{100} 80 μM) are effective against mammalian tyrosine kinases (Fan *et al.*, 1995), whereas GW 5074 (IC_{100} 60 μM) inhibits the cRaf-1 kinase (Lackey *et al.*, 2000), which functions downstream of the Ras GTPase. Identification of L-744,832, a known farnesyltransferase and Ras inhibitor (Barrington *et al.*, 1998), as a BHT inhibitor, together with GW 5074, reinforces the central role that the Ras signalling pathway plays in regulating the BHT (Leberer *et al.*, 2001).

Identification of the dopamine G protein-coupled receptor (GPCR) antagonists clozapine (IC_{100} 50 μM) and fluspirilene (IC_{100} 40 μM) as BHT inhibitors was very interesting, especially given that clozapine and a number of its bioactive derivatives are FDA approved for the treatment of atypical schizophrenia (Ereshefsky *et al.*, 1989). There are only three annotated GPCRs in *C. albicans*, with two being the STE2 and STE3 pheromone receptors. The remaining GPCR is Gpr1p, which has been implicated in a nutrient-regulated BHT signalling pathway upstream of PKA (Maidan *et al.*, 2005; Miwa *et al.*, 2004). It remains to be determined whether clozapine and fluspirilene function through the Gpr1p GPCR.

The BHT inhibitors CGP-37157 (IC_{100} 40 μM), TMB-8 (IC_{100} 40 μM), FK506 (IC_{100} 60 μM), W7 (IC_{100} 40 μM), HA14-1 (IC_{100} 20 μM) and nigericin (IC_{100} 130 μM) are known to affect calcium homeostasis in the cell, by affecting calcium/calmodulin-dependent protein kinases (W7; Kawamoto & Hidaka, 1984), the calcineurin phosphatase (FK506; Schreiber & Crabtree, 1992) or intracellular calcium levels (CGP-37157, Cox *et al.*, 1993; TMB-8, Chiou & Malagodi, 1975; nigericin, Vercesi *et al.*, 1993; and HA14-1, Degterev *et al.*, 2001). Although FK506 does not inhibit the growth of *C. albicans* cells, it has been shown to display synergistic effects with the known azole antifungal drug fluconazole (Uppuluri *et al.*, 2008). Cyclosporine A also showed this synergy, but did not inhibit the BHT on its own (data not shown), as FK506 did.

Interesting, HA14-1 has also been shown to be an inducer of apoptosis in mammalian cells (Degterev *et al.*, 2001). However, several other inducers of apoptosis in the collection (C8 ceramine, 5d-prostaglandin J2, lysophosphatidic acid and Y-27632) were not cytotoxic and did not inhibit the BHT (data not shown). Apoptosis is regulated in mammalian cells by the Bcl-2 family proteins Bcl-2, Bcl-lx, Bak and Bax (Danial & Korsmeyer, 2004). HA14-1 induces apoptosis by specifically binding to the Bcl-2 homology 3 domain of Bcl-2, thereby preventing the antagonistic interaction between Bcl-2 and the Bak/Bax proteins. Unfortunately, there are no recognizable Bcl-2 family proteins encoded in fungal genomes (Fedorova *et al.*, 2005; Madeo *et al.*, 2002); therefore, the target of HA14-1 and its mechanism of action in BHT inhibition remain to be elucidated.

BHT inhibitors block conidial germination and hyphal formation in *A. fumigatus* and other *Candida* species

The effects of 11 of the 16 BHT inhibitors (excluding tyrphostin 9, L-744,832, fluspirilene, ETYA and nigericin) on conidial germination and hyphal elongation in the pathogenic filamentous fungus *A. fumigatus* were also examined. *Aspergillus* species are second only to *Candida* species in causing systemic human fungal infections, with *A. fumigatus* being the most prevalent cause of human aspergillosis (Warnock, 2007). We observed that addition of clozapine and CGP-37157 blocked conidia germination and hyphae formation, whilst addition of GW 5074, HA14-1, FK506, W7 and YC-1 had partial effects after an 8 h incubation (Fig. 1a). Incubation with HA14-1, FK506, W7 and YC-1 for an additional 6 h showed significant conidial germination and hyphal elongation, suggesting that these molecules were not effective inhibitors (data not shown). The effects of clozapine and CGP-37157 were reversible, as they are in *C. albicans*, and both molecules could also inhibit hyphal elongation following conidial germination (data not shown).

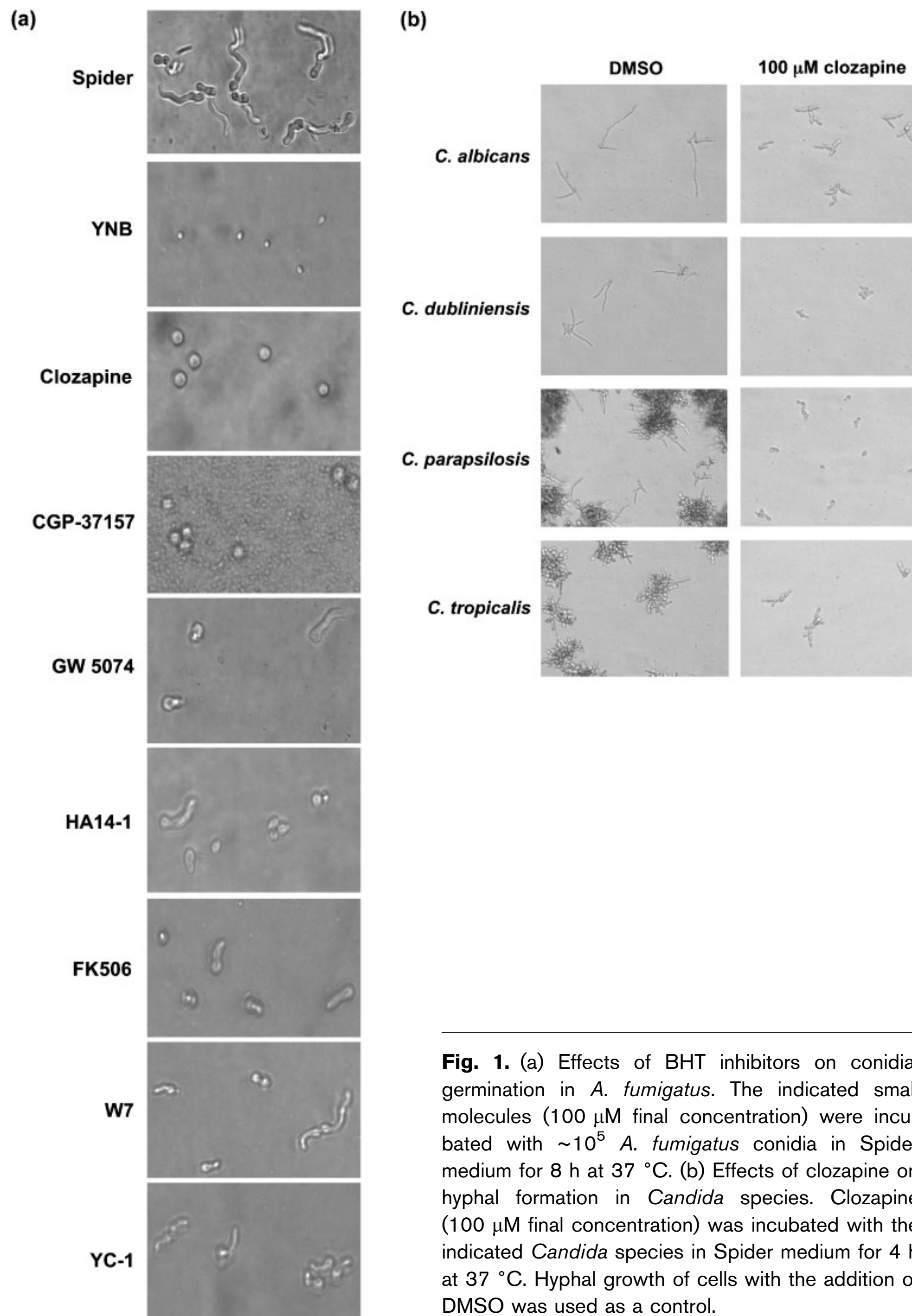

Fig. 1. (a) Effects of BHT inhibitors on conidial germination in *A. fumigatus*. The indicated small molecules (100 μM final concentration) were incubated with $\sim 10^5$ *A. fumigatus* conidia in Spider medium for 8 h at 37 °C. (b) Effects of clozapine on hyphal formation in *Candida* species. Clozapine (100 μM final concentration) was incubated with the indicated *Candida* species in Spider medium for 4 h at 37 °C. Hyphal growth of cells with the addition of DMSO was used as a control.

The same 11 molecules were examined for their effects on morphogenesis in other *Candida* species. Only three molecules, clozapine (Fig. 1b), HA14-1 and ETYA (data not shown), inhibited filamentous growth in *Candida dubliniensis*, *Candida parapsilosis* and *Candida tropicalis*. Taken together, these results suggested that a subset of the BHT inhibitors, including clozapine, may have a broad host range among yeast and filamentous fungi, and hence may be better starting candidates for antifungal therapeutics.

Effects of BHT inhibitors on hyphae-associated cellular processes

The effects of 13 of the 16 BHT inhibitors (excluding L-744,832, ETYA and nigericin) on the expression of the

hyphal-specific *HWP1* pr–GFP reporter gene were determined. Previously, we showed that expression of the *HWP1* pr–GFP reporter gene was strongly induced under hypha-inducing conditions, such as growth in Spider medium, and that small-molecule BHT inhibitors could have differential effects on *HWP1* pr–GFP expression (Toenjes *et al.*, 2005). Of the 13 BHT inhibitors tested, only GW 5074-treated cells showed appreciable fluorescence (Fig. 2). This result suggested that the remaining 12 molecules tested inhibited the expression of *HWP1* pr–GFP, possibly by inhibiting a component of the *EFG1*, *NRG1* or *TUP1* transcriptional pathway that regulates *HWP1* expression. GW 5074 inhibits mammalian c-Raf kinase, which functions downstream of the Ras GTPase. In *C. albicans*, Ras is believed to signal through the *EFG1* transcription factor pathway (Biswas *et al.*, 2007), so it is not clear why

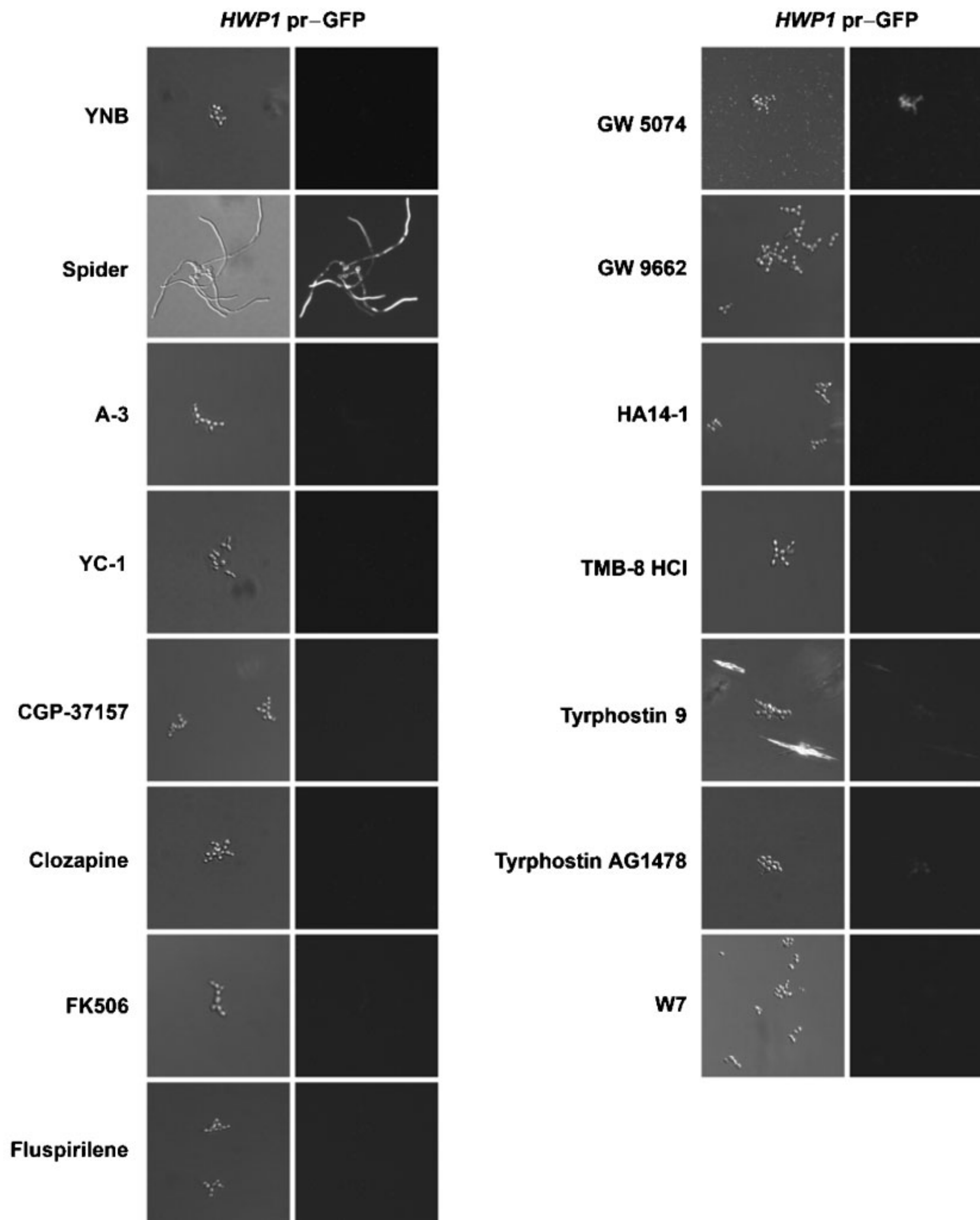

Fig. 2. Effects of BHT inhibitors on *HWP1* pr–GFP expression. The indicated small molecules (100 μM final concentration) were incubated with KTCa1 cells in Spider medium for 4 h at 37 °C.

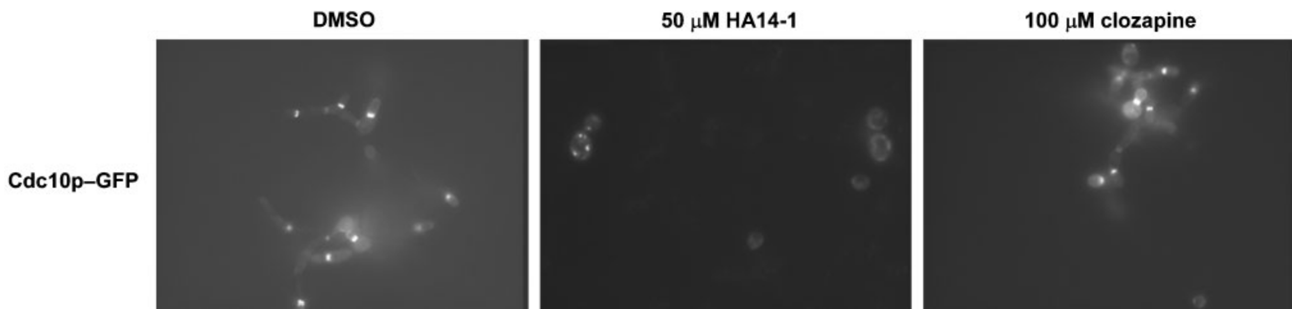

Fig. 3. Effects of BHT inhibitors on septin localization. HA14-1 (50 μM final concentration) and clozapine (100 μM final concentration) were incubated with YAW2 cells in Spider medium for 4 h at 37 °C. Addition of HA14-1 resulted in abnormal septin localization, whereas addition of clozapine had no effect. Addition of DMSO was used as a control.

HWP1 pr–GFP expression was observed in GW 5074-treated cells; an alternative target for GW 5074 is a likely possibility.

The effects of the bioactive molecules on septin ring assembly were also examined. Septins play an important role in cellular morphogenesis and polarized growth in *C. albicans* and most other eukaryotes (Warenda *et al.*, 2003). YAW2 cells expressing a septin Cdc10p–GFP fusion protein were used to examine septin organization. Of the nine BHT inhibitors tested (YC-1, tyrphostin 9, HA14-1, GW 5074, FK506, GW 9662, fluspiriline, clozapine and TMB-8), only HA14-1 showed a defect in the localization of the Cdc10p–GFP fusion protein (Fig. 3). It remains to be determined whether this abnormal septin localization is a cause of, or the consequence of, BHT inhibition.

The molecules identified in this study will hopefully prove to be a useful and informative resource, not only for new antifungal therapeutics but also as tools to decipher the signal-transduction pathways that regulate cell growth and the BHT in *C. albicans*. The fact that several of these molecules also affected morphogenetic processes in other *Candida* species and in the filamentous fungus *A. fumigatus* raises the exciting possibility of a broad fungal host range for these molecules, as well as conservation of function for the affected signalling pathways and the targets of the molecules in both budded and filamentous fungal species.

ACKNOWLEDGEMENTS

We thank Gary Ward, James Konopka and Kieren Marr for valuable reagents, the ICCB-Longwood Screening Facility for the BIOMOL–ICCB Known Bioactives collection, Ted Wilson and Dan Reichert for technical support, and Gary Ward, David K. Butler and members of the Johnson and Toenjes laboratories for valuable discussions and critical comments on this manuscript. This research was supported in part by USDA-Hatch grant VT-H01308 from the Vermont Experiment Station (D. I. J.), and NIH grant P20 RR16455-04 from the INBRE Program of the National Center for Research Resources (K. A. T.).

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