**This is a pre- or post-print of an article published in
Heintz-Buschart, A., Eickhoff, H., Hohn, E., Bilitewski,
U.**

**Identification of inhibitors of yeast-to-hyphae
transition in *Candida albicans* by a reporter screening
assay**

(2013) Journal of Biotechnology, 164 (1), pp. 137-142.

Identification of inhibitors of yeast-to-hyphae transition in *Candida albicans* by a reporter screening assay

Anna Buschart^a, Holger Eickhoff^b, Erwin Hohn^b, Ursula Bilitewski^{a*}

^aHelmholtz Centre for Infection Research, Department of Biological Systems Analysis,
Inhoffenstr.7, 38124 Braunschweig, Germany

^bEMC microcollections GmbH, Sindelfinger Str. 3, 72070 Tübingen, Germany

***Corresponding author:** Ursula Bilitewski, Biological Systems Analysis, Helmholtz Centre for Infection Research, Inhoffenstr. 7, 38124 Braunschweig, Germany, e-mail:
ursula.bilitewski@helmholtz-hzi.de, tel: +49-531-6181-1010, fax: +49-531-6181-1096

Running title: High-throughput assay for hyphal inhibitors in *C. albicans*

ABSTRACT

Candida albicans is one of the most common opportunistic fungal pathogens, causing life-threatening disease in immunocompromised patients. As it is not primarily a pathogen, but can exist in a commensal state, we aimed at the identification of new anti-infective compounds which do not eradicate the fungus, but primarily disable a virulence determinant. The yeast-hyphae-dimorphism of *C. albicans* is considered a major contributor to fungal disease, as mutants locked into either yeast or hyphal state have been shown to be less virulent in the mouse-model.

We devised a high-throughput screening procedure which allows us to find inhibitors of the induction of hyphae. Hyphae-formation was induced by nitrogen starvation at 37 °C and neutral pH in a reporter strain, which couples promotor activity of the hyphae-specific *HWP1* to β -galactosidase expression. In a pilot screening of 720 novel synthetic compounds, we identified substances which inhibited the outgrowth of germ tubes. They belonged to chemical classes not yet known for antimycotic properties, namely methyl aryl-oxazoline carboxylates, dihydrobenzo[d]isoxazolones and thiazolo[4,5-e]benzoxisoxazoles.

In conclusion we developed a novel screening assay, which addresses the morphological switch from the yeast form of *C. albicans* to its hyphal form and identified novel chemical structures with activity against *C. albicans*.

Keywords: reporter strain; beta-galactosidase; HWP1; morphology

Abbreviations:

HWP – hyphal wall protein

MTT - 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide

ONPG – *o*-nitrophenyl- β -D-galactoside

YNB – yeast nitrogen base

SLAD – synthetic low ammonium dextrose

1. Introduction

With the progress in intensive medicine, fungal infections have become a serious public health problem, as advanced therapies of severe diseases are successfully applied, but render more patients with predisposing factors for fungal infections. These include a compromised immune status, central venous catheters, gastrointestinal surgery and extremes of age (Perlroth et al., 2007; Pfaller and Diekema, 2007). The most common cause of invasive mycoses is the polymorphic yeast *Candida albicans*, an opportunistic pathogen which is found in its commensal state on the mucosa of the majority of the healthy population without causing disease symptoms. Systemic candidiasis, however, can be lethal and accounts for a mortality rate of 30-50 % (Tortorano et al., 2004; Wisplinghoff et al., 2004). In addition, *C. albicans* can cause superficial infections, e.g. of the mucosa of mouth and vagina, which are not life-threatening but significantly reduce the quality of life.

In spite of the clinical application of four classes of antimycotics against systemic candidiasis, there have been continuous efforts to discover and develop new antimycotic compounds, as existing drugs may be associated with toxic side-effects or interactions with other therapeutics, high costs and resistance development (Gubbins and Amsden, 2005; Vermes et al., 2000). Beside the screening for compounds with fungicidal or fungistatic activity and the description of novel potential antimycotic therapeutics (reviewed by Calugi et al. (2011)), the recent years also saw the development of novel approaches to the search for antifungal agents (for example Burger-Kentischer et al., 2011; Lafleur et al., 2011).

One recent approach is the search for new lead compounds which attenuate virulence factors, the so-called pathoblockers. Resistance development to virulence-attenuating compounds should be rare, as the selective pressure on the population would not be as great, since avirulent subpopulations can survive (Jiang et al., 2002). The yeast-hyphae-dimorphism is one of the most important and well-known virulence factors in *C. albicans*. Mutants locked into either the yeast or the filamentous morphology are avirulent in infection models (Lo et al., 1997; Murad et al., 2001b). The morphological change also influences other virulence factors, such as adhesion and biofilm formation. Moreover, yeast cells are more easily distributed in the bloodstream, and hyphae allow penetration of host tissue to access nutrient sources. The switch to hyphal growth can also help *C. albicans* to escape from phagosomes (Arai et al. 1977).

Cultivation in rich media with a low pH at temperatures up to 30 °C favours growth as yeasts, whereas hyphal formation is triggered by nitrogen starvation, the presence of serum and 5 % CO₂, and an increase of temperature to 37 °C and a neutral pH (Mitchell and Soll, 1979).

Numerous signal transduction pathways control the switch between hyphae and yeast morphology. Under yeast favouring conditions, the expression of hyphae-specific genes is controlled by a set of transcriptional repressors (Murad et al., 2001a, 2001b). The cAMP-protein kinase A-pathway plays a central role in the induction of hyphae: the adenylate cyclase Cyr1p is activated by a number of stimuli, such as CO₂-concentration, and signals from other proteins, e.g. the small G-protein Ras1p and the cytoskeleton (Fang and Wang, 2006; Klengel et al., 2005; Rocha et al., 2001; Zou et al., 2010). Ras1p itself is activated in response to multiple signals, like nitrogen shortage or the presence of serum (Biswas and Morschhäuser, 2005; Feng et al., 1999). The resulting increase in cAMP-concentration activates protein kinase A (Bockmühl et al., 2001), which in turn activates the transcription factor Efg1p to induce the expression of hyphae-specific genes, either alone or in interaction with further transcriptional activators (Lane et al., 2001; Schweizer et al., 2000; Stoldt et al., 1997). Besides the cAMP-protein kinase A-pathway, MAP kinase-pathways are also involved in the induction of hyphae (Köhler and Fink, 1996; Leberer et al., 1996). The MAP kinases Cek2p and, more importantly, Cek1p are activated via the MAP kinase kinase Hst7p and the MAP kinase kinase kinase Cst20p (Leberer et al., 1996; Csank et al., 1998). Cst20p can be activated by Ras1p, like Cyr1p (Köhler and Fink, 1996). The activation of these MAP kinases leads to the induction of hyphae-specific genes by the transcription factor Cph1p (Liu et al., 1994). Extracellular pH is another important morphogenetic stimulus, and is signalled via the Rim-pathway. Rim101p is a transcription factor inhibiting expression of hyphae-specific genes. At neutral pH, a number of Rim-proteins, as well as the ESCRT-complex, are recruited to the pH-sensor Rim20p (Xu and Mitchell, 2001). This triggers the proteolytic cleavage of Rim101p, turning Rim101p into an activator of hyphae-specific gene expression (Li et al., 2004).

Amongst the hyphae specific genes is *HWPI*. It codes for the adhesin Hwp1p, which was detected in the cell wall of hyphae but never of blastoconidia. Likewise, the *HWPI*-transcript is only present after commitment to hyphal growth (Staab et al., 1996, 1999).

We aimed for a potential high-throughput screening to find compounds which inhibit formation of hyphae in *C. albicans*. To this end, we made use of a reporter construct developed by Uhl and Johnson (2001) in which the promoter of *HWPI* was fused to the gene for β -galactosidase from *Streptococcus thermophilus*. Hyphal induction of *C. albicans* CAI-4 transformed with this construct can be determined via measurement of the activity of β -galactosidase (Uhl and Johnson, 2001; Hogan et al., 2004). By adapting the β -galactosidase assay to automated use in microtitre plate formats, we were able to identify three novel classes of antimycotic compounds in a pilot screening.

2. Materials and methods

2.1 Preparation of test compounds

All test compounds were dissolved in DMSO. MDL-12,330A (*cis*-N-2-(phenyl-cyclopentyl)-azacyclotridec-1-en-2-amine) was obtained from Sigma and a 5 mM stock solution was prepared. Concentrations of stock solutions for farnesol (Sigma) were 1.1 mM and 1 g/l for the 720 novel synthetic compounds from EMC microcollections GmbH, Tübingen, Germany.

2.2 Strains and culture conditions

Candida albicans CAI-4 *HWPI-lacZ* (Hogan et al., 2004) was used in this study. Integration of *lacZ* upstream of the *HWPI*-promotor was confirmed by sequencing products of an uneven PCR executed according to the method of Chen and Wu (1996). Cultures inoculated from a cryo-stock were grown overnight in a defined medium (containing 6.7 g/l yeast nitrogen base without amino acids, 9 g/l glucose and 1 g/l maltose) at 30 °C. Cells from the preculture were washed twice in pre-warmed hyphal induction medium SLAD+ (1.7 g/l yeast nitrogen base without amino acids and without ammonium sulfate, 2 g/l glucose, 1 g/l maltose, 6 mg/l ammonium sulfate, buffered to pH 7.3 using 0.165 M MOPS) and afterwards resuspended in the same medium. The OD_{620nm} was adjusted to 0.05 to 0.1 as determined using a microtitre plate reader (µQuant, Biotek) in 180 µl sample volume (approx. 4-8*10⁶ cells/ml). 50 µl of this suspension were added to each well of a 96-well plate. 1 µl of each test compound or DMSO as solvent-control were added. The microtitre plate was incubated at 37 °C for 3h to induce hyphal growth.

2.3 Assay for β-galactosidase activity

After the induction of hyphal growth, *C. albicans* cells were lysed by the addition of 100 µl z-buffer, which was adapted from Kippert (1995) (60 mM Na₂HPO₄, 40 mM NaH₂PO₄, 10 mM KCl, 1 mM Mg₂SO₄, 1 mM DTT, 0,2 % sodium lauroyl sarcosinate) to the *C. albicans* suspension in each well. Lysis was performed at 37 °C for 40 min.

Afterwards, 50 µl of 4 g/l ONPG (*o*-nitrophenyl-β-D-galactoside) in 0.1 M potassium phosphate buffer (pH 7.0) were added to each well. ONPG was hydrolysed by β-galactosidase during 90 min incubation at 37 °C. Optical densities at 414 nm and 550 nm were determined.

2.4 Data analysis

Calculation of β-galactosidase activity was adjusted from the calculation of Miller units (equation 1), which takes into account the absorption by *o*-nitrophenol produced from ONPG, the incubation

time, the culture volume and OD_{600 nm} of the culture (Miller, 1972). Between the assays on the same microtitre plate, the incubation time, starting volume and optical density were kept constant. Therefore, the absorption by *o*-nitrophenol (which we measured at the absorption maximum 414 nm) produced by hydrolysis of ONPG could serve as a measure of β -galactosidase activity. To eliminate the scatter by cell debris, Miller (1972) suggested an additional measurement at 550 nm, where *o*-nitrophenol absorption is low. He introduced a factor (designated *c* in eqn 1) to be multiplied with the OD_{550 nm} in order to approximate the contribution of the scatter by cell debris to the OD measured at 414 nm from the measurement at 550 nm. We experimentally determined *c* to be 1.36 when using microtitre plates instead of standard 1 cm-cuvettes our set-up. The absorption by *o*-nitrophenol was therefore calculated according to equation 1.

Equation 1: absorption by *o*-nitrophenol = OD_{414 nm} - *c* * OD_{550 nm}

2.5 High-throughput assay

The high-throughput assay was performed as described above, with all pipetting steps automated on an Evolution P³ with Plate Stak (Perkin Elmer) automatic pipetting platform. In a pilot screening, 720 novel synthetic compounds from EMC microcollections GmbH at a final concentration of 20 mg/l (2 % DMSO) were tested. Measurements were performed on a microtitre plate reader (Fusion, Perkin Elmer) using filters centred around 425 nm and 550 nm. As some of the compounds had a strong yellow colour, the plates were also measured immediately after the addition of ONPG and these values were subtracted from the measurements after 90 min, according to equation 2 (with *c* being 1 in our plate reader).

Equation 2: absorption by *o*-nitrophenol = (OD_{425nm} - *c* * OD_{550nm})_{90min} - (OD_{425nm} - *c* * OD_{550nm})_{0min}

The ratio of this value to the mean of the negative controls (solvent controls) was calculated. The screening was repeated three times.

As a measure of assay quality control, the Z'-factor was calculated from the standard deviations (σ) and means (μ) of the positive (p) and negative (n) controls, according to equation 3 (from Zhang et al., 1999):

Equation 3: $Z' = 1 - 3 * (\hat{\sigma}_p + \hat{\sigma}_n) / |\hat{\mu}_p - \hat{\mu}_n|$

2.6 IC₅₀ determination of HWPI-expression

The assay was performed as described above, but instead of a single concentration a dilution series of the test compound was added to in the induction medium. The assay was performed in half area plates (Corning) with culture volumes and the respective volumes of lysis buffer and ONPG-solution half as large as described above.

2.7 Susceptibility testing under yeast-favouring conditions

Growth inhibitory effects of antimycotic compounds on *C. albicans* yeast cells in liquid culture were determined in microtitre plates (180 µl volume). *C. albicans* was cultured over night in 50 ml YPD medium. A pre-culture was set up in YNB (6.7 g/l yeast nitrogen base, 20 g/l glucose) and 5000 cells/ml from an exponentially growing pre-culture were incubated in YNB at 30 °C with a dilution series of the compounds for 24 h and growth was determined photometrically by measuring the OD_{620nm}. Experiments were conducted in triplicate cultures.

2.8 Cytotoxicity

The human epithelial cell line A549 (Giard et al., 1973; Lieber et al., 1976) was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultivated in DMEM (Lonza) complemented with 10 % foetal calf serum (FBS “Gold“, PAA Laboratories GmbH, Pasching, Austria) in 25 cm² tissue culture flasks at 37 °C and 5 % CO₂.

To determine the cytotoxicity of the novel compounds, 6000 cells per well were seeded in 96-well plates (180 µL) and incubated with dilution series of the compounds for 5 days at 37 °C and 5 % CO₂. Final concentrations ranging from 0.01 to 60 mg/l were tested.

After 5 days, 500 mg/l MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide, from Sigma, dissolved in PBS at a stock concentration of 5 g/l) was added and incubation was continued for 2 h. The medium was discarded by inversion and the cells were washed once with 100 µl PBS, which was also discarded. 100 µl Isopropanol-HCl (0,4 % (v/v) 32 % HCl) were added to each well and incubated at room temperature for 20 min, before the absorption at 595 nm was measured. The ratios of these values to the corresponding values from a solvent control were calculated.

Experiments were performed in triplicate cultures.

3. Results

3.1 Establishment of β -galactosidase activity assay

As numerous environmental factors can induce and inhibit hyphal growth of *C. albicans*, we sought

to establish well-defined culture conditions that would lead to reproducible outgrowth of germ tubes in our test strain. The inherent neutral pH and low nutrient availability of SLAD+ medium, as well as the elevated temperature (37 °C), constituted such conditions. In addition, SLAD+ is, in contrast to serum as an often used hyphal inductor, a defined medium and does not cause formation of froth, which is problematic in automated liquid handling. The medium was buffered so that the pH of the medium was not affected by acidic or basic test compounds.

Induction cultures were inoculated with *C. albicans* from the early stationary phase, as hyphal outgrowth could be efficiently induced in these cells (fig S1 A). As the formation of hyphae is limited by cell density in *C. albicans*, we tested the effect of this factor on the *HWPI*-reporter assay. Cell densities of $4-8 \times 10^6/\text{ml}$ achieved maximal rates of substrate turn-over (fig S1 B).

The induction time of 3 h was chosen as *HWPI* expression was high at this point in time (fig S1 C) and to ease optional microscopic inspection, as germ tubes were clearly visible after this time.

Reaction times for cell lysis and β -galactosidase reaction were chosen in accordance to previously published protocols (Kippert, 1995; Menzel, 1989; Miller, 1972) and to allow convenient handling time and parallel performance of large numbers of assays. The hydrolysis of ONPG in solvent controls remained proportional to the reaction time (fig S1 D), demonstrating that the reaction was not yet saturated after 1.5 h.

As low nitrogen availability was one of the hyphae-inducing factors in this assay, we wanted to exclude the possibility that test compounds could serve as nitrogen sources and, hence, influence the test result. Taking into account the molecular weights of the test substances, their potential nitrogen content and the test concentration of 20 mg/l, we added ammonium sulfate, a preferred nitrogen source of *C. albicans*, in concentrations up to 150 μM . We did not observe any changes in *HWPI*-expression (fig S1 E).

The effects of known inhibitors of hyphae induction were tested to evaluate the assay's performance. MDL-12,330A is a specific inhibitor of adenylate cyclase Cyr1p. As Cyr1p is a central regulator of hyphae development in *C. albicans*, MDL-12,330A consequently inhibits hyphal growth (Sato et al., 2004). Treatment of *C. albicans HWPI-lacZ* with 100 μM MDL-12,330A caused a significant reduction of hyphal growth, as was visible by microscopic analysis, as well as of β -galactosidase activity (fig 1 and fig S1 F).

3.2 Validation of high-throughput assay

Likewise, the quorum sensing compound farnesol is a potent inhibitor of hyphal development (Hornby et al., 2001). At a concentration of 22.5 μM , it inhibited nearly all germ tube formation and almost completely abolished β -galactosidase activity (fig 1). Farnesol was used as positive control

to calculate the Z'-factor (Zhang et al., 1999; eqn 3) of the automated high-throughput assay. *C. albicans* was incubated with 22.5 μ M farnesol (positive control) or 2 % DMSO as solvent (negative) control as described using the liquid handling station. The results from 48 positive and 48 negative controls are shown in fig. 1 and the Z'-factor was calculated to be 0.78, which indicates an excellent assay (according to Zhang et al., 1999). The coefficient of variation for the negative (solvent) controls was 0.06.

3.3 Screening of 720 synthetic compounds and retesting

In a pilot screening campaign we tested 720 novel compounds, which were synthesised by EMC microcollections GmbH, in the automated *HWPI*-reporter assay at a final concentration of 20 mg/l. This concentration was chosen high to detect also weakly active compounds from among the as yet uncharacterised collection. We repeated the test three times with all compounds and performed a manual retest of those compounds, which reduced β -galactosidase activity by at least 90 % in all three screenings. We identified 10 compounds which reproducibly inhibited hyphal formation significantly (tab 1, fig 2). These 10 compounds belonged to three chemical classes, namely methyl aryl-oxazoline carboxylates, dihydrobenzo[d]isoxazolones and thiazolo[4,5-e]benzoxazoles (fig 2). None of these compounds or compound classes were previously known to be antimycotic or to inhibit hyphal development.

A range of concentrations of the identified compounds was tested for hyphae-inhibiting activity. The IC₅₀ values for *HWPI*-expression as detected by the β -galactosidase assay were in the micromolar range (tab 1).

3.4 Inhibition of growth of *C. albicans* SC5314

As fungicidal and fungistatic compounds can also cause a decrease in *HWPI*-expression, the fungistatic potential of the hit compounds against *C. albicans* in yeast morphology was assessed. The chosen test conditions reflected similarities to the hyphae-inducing conditions in the choice of medium base (yeast nitrogen base), but offered large amounts of nutrients (glucose and ammonium sulfate), an acidic pH and lower temperature to ensure yeast growth. Growth in the presence of a dilution series of the compounds was determined photometrically and the IC₅₀-values are displayed in table 1. For most compounds, the critical concentrations for inhibition of *HWPI*-expression and yeast growth were similar or identical. Therefore, these compounds probably did not inhibit hyphal growth by specific inhibition of the respective signal transduction cascades.

However, compound 10 and – to a lesser extent – compound 8 inhibited germ tube formation at lower concentrations than yeast growth.

3.5 Cytotoxicity

As cytotoxicity against human cells is undesirable in potential therapeutics, the cytotoxicity of the hit compounds at different concentrations was determined in 5 d exposure tests using the human epithelial cell-line A549. Most compounds showed cytotoxic activity within the same concentration range as the antimicrobial activity, regardless of the chemical class of the compound. The compounds 2, 4, 7 and 8 were less toxic to human cells than to *C. albicans* (tab 1).

4. Discussion

Fungal infections are amongst the most common complications of intensive medicine and *C. albicans* is the most frequent fungal pathogen (Pfaller and Diekema, 2007; Tortorano, 2004). In addition to severe illness, it can cause superficial infections of the mucosa which are very frequent. Treatment of these infections relies on the availability of potent and safe anti-infectives. Drug discovery should exploit the continuous improvement of the understanding of the factors that lead to pathogenicity of this opportunistic fungus.

While a traditional approach to lead structure discovery was the screening of large libraries of compounds for fungistatic or fungicidal activity by detecting growth under *in vitro* conditions which did not take into account the understanding of the infection process, the recent years have seen the advent of new approaches. Especially with respect to opportunistic pathogens, the present understanding is that the mere presence of the microbe is not in itself harmful. Therefore the eradication of the microbe must not be the only way to treat an infection, but attenuators of virulence traits were searched (Jiang, 2002). Thus, the development of novel tools for screening for compounds with therapeutic potential which target pathogenicity rather than viability is desirable. We present here a novel screening assay, which makes use of a reporter strain to detect inhibitors of hyphal development. The morphological switch between yeast and hyphae is considered one of the most important virulence traits of *C. albicans* (Calderone and Fonzi, 2001), and disabling this switch abolished virulence in infection models (Saville et al., 2006). A number of chemically unrelated compounds are already known to inhibit the yeast-to-hyphae transition of *C. albicans* and the first applications of these compounds to infection models have been published, showing protective effects in models of mucosal infections (Akerey et al., 2009; Hisajima et al., 2008; Shareck and Belhumeur, 2011). In our approach promoter activity of the hyphae-specific *HWP1* gene is detected by means of a β -galactosidase assay. This approach is similar to a method described

by Jarosz and Krom (2011) using a GFP-reporter assay. However, due to the greater sensitivity and robustness of the enzymatic assay, we could optimise our assay for automation and a higher throughput.

Optimised for automated liquid handling in miniaturised volumes, the assay presented here can be carried out in line with standard yeast growth assays and toxicity tests. A pilot screening yielded inhibitors of yeast growth and hyphal formation of *C. albicans* from three chemical classes, methyl aryl-oxazoline carboxylates, dihydrobenzo[d]isoxazolones and thiazolo[4,5-e]benzoxisoxazoles.

These classes of compounds were not yet known to inhibit fungal growth or influence fungal morphology. The active compounds with the lowest toxicity on mammalian epithelial cells (compounds 7 and 8) were aryl-oxazoline-carboxylates. An aryl-oxazoline-carboxylate was previously discovered as an antibacterial agent against Gram-positive species (Sasaki et al., 1997).

To conclude, we show that this novel assay can be a valuable tool for the discovery of novel inhibitors of the yeast-hyphal switch, which could be developed as anti-infectives or whose mechanism of action will shed further light on the processes involved in yeast-to-hyphae transition.

Acknowledgements

The authors thank Deborah Hogan (Department of Microbiology and Immunology, Dartmouth Medical School, New Hampshire, USA) for kindly providing us with *C. albicans* *HWPI-lacZ*. We also thank Florenz Sasse for providing screening facilities and fruitful discussions. This work was supported by the German Ministry of Research – project “Novel drugs against fungal human pathogens” (grant number 0315221A to A.B.).

References

- Akerey, B., Le-Lay, C., Fliss, I., Subirade, M., Rouabhia, M., 2009. In vitro efficacy of nisin Z against *Candida albicans* adhesion and transition following contact with normal human gingival cells. J. Appl. Microbiol. 107, 1298-307.
- Arai T., Mikami, Y., Yokoyama, K., 1977. Phagocytosis of *Candida albicans* by rabbit alveolar macrophages and guinea pig neutrophils. Sabouraudia 15, 171-7.

- Biswas, K., Morschhäuser, J., 2005. The Mep2p ammonium permease controls nitrogen starvation-induced filamentous growth in *Candida albicans*. *Mol. Microbiol.* 56, 649-69.
- Bockmühl, D.P., Krishnamurthy, S., Gerads, M., Sonneborn, A., Ernst, J.F., 2001. Distinct and redundant roles of the two protein kinase A isoforms Tpk1p and Tpk2p in morphogenesis and growth of *Candida albicans*. *Mol. Microbiol.* 42, 1243-57.
- Burger-Kentischer, A., Finkelmeier, D., Keller, P., Bauer, J., Eickhoff, H., Kleymann, G., Abu Rayyan, W., Singh, A., Schröppel, K., Lemuth, K., Wiesmüller, K.H., Rupp, S., 2011. A screening assay based on host-pathogen interaction models identifies a set of novel antifungal benzimidazole derivatives. *Antimicrob. Agents Chemother.* 55, 4789-801.
- Calderone, R.A., Fonzi, W.A., 2001. Virulence factors of *Candida albicans*. *Trends. Microbiol.* 9, 327-35.
- Calugi, C., Trabocchi, A., Guarna, A., 2011. Novel small molecules for the treatment of infections caused by *Candida albicans*: a patent review (2002-2010). *Expert Opin. Ther. Pat.* 21, 381-97.
- Chen, X., Wu, R., 1996. Direct amplification of unknown genes and fragments by Uneven polymerase chain reaction. *Gene* 185, 195-9.
- Csank, C., Schröppel, K., Leberer, E., Harcus, D., Mohamed, O., Meloche, S., Thomas, D.Y., Whiteway, M., 1998. Roles of the *Candida albicans* mitogen-activated protein kinase homolog, Cek1p, in hyphal development and systemic candidiasis. *Infect. Immun.* 66, 2713-21.
- Fang, H.M., Wang, Y., 2006. RA domain-mediated interaction of Cdc35 with Ras1 is essential for increasing cellular cAMP level for *Candida albicans* hyphal development. *Mol. Microbiol.* 61, 484-96.
- Feng, Q., Summers, E., Guo, B., Fink, G., 1999. Ras signaling is required for serum-induced hyphal differentiation in *Candida albicans*. *J. Bacteriol.* 181, 6339-46.
- Giard, D.J., Aaronson, S.A., Todaro, G.J., Arnstein, P., Kersey, J.H., Dosik, H., Parks, W.P., 1973. In

vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. J. Natl. Cancer. Inst. 51, 1417-23.

Gubbins, P.O., Amsden, J.R., 2005. Drug-drug interactions of antifungal agents and implications for patient care. Expert Opin. Pharmacother. 6, 2231-43.

Hisajima, T., Maruyama, N., Tanabe, Y., Ishibashi, H., Yamada, T., Makimura, K., Nishiyama, Y., Funakoshi, K., Oshima, H., Abe, S., 2008. Protective effects of farnesol against oral candidiasis in mice. Microbiol. Immunol. 52, 327-33.

Hogan, D.A., Vik, A., Kolter, R., 2004. A *Pseudomonas aeruginosa* quorum-sensing molecule influences *Candida albicans* morphology. Mol. Microbiol. 54, 1212-23.

Hornby, J.M., Jensen, E.C., Lisec, A.D., Tasto, J.J., Jahnke, B., Shoemaker, R., Dussault, P., Nickerson, K.W., 2001. Quorum sensing in the dimorphic fungus *Candida albicans* is mediated by farnesol. Appl. Environ. Microbiol. 67, 2982-92.

Jarosz, L.M., Krom, B.P., 2011. Rapid screening method for compounds that affect the growth and germination of *Candida albicans*, using a real-time PCR thermocycler. Appl. Environ. Microbiol. 77, 8193-6.

Jiang, B., Bussey, H., Roemer, T., 2002. Novel strategies in antifungal lead discovery. Curr. Opin. Microbiol. 5, 466-71.

Kippert, F., 1995. A rapid permeabilization procedure for accurate quantitative determination of beta-galactosidase activity in yeast cells. FEMS Microbiol. Lett. 128, 201-6.

Klengel, T., Liang, W.J., Chaloupka, J., Ruoff, C., Schröppel, K., Naglik, J.R., Eckert, S.E. Mogensen, E.G., Haynes, K., Tuite, M.F., Levin, L.R., Buck, J., Mühlischlegel, F.A., 2005. Fungal adenylyl cyclase integrates CO₂ sensing with cAMP signaling and virulence. Curr. Biol. 15, 2021-6.

Köhler, J.R., Fink, G.R., 1996. *Candida albicans* strains heterozygous and homozygous for mutations in mitogen-activated protein kinase signaling components have defects in hyphal development. Proc. Natl. Acad. Sci. USA 93, 13223-8.

- Lafleur, M.D., Lucumi, E., Napper, A.D., Diamond, S.L., Lewis, K., 2011. Novel high-throughput screen against *Candida albicans* identifies antifungal potentiators and agents effective against biofilms. *J. Antimicrob. Chemother.* 66, 820-6.
- Lane, S., Birse, C., Zhou, S., Matson, R., Liu, H., 2001. DNA array studies demonstrate convergent regulation of virulence factors by Cph1, Cph2, and Efg1 in *Candida albicans*. *J. Biol. Chem.* 276, 48988-96.
- Leberer, E., Harcus, D., Broadbent, I.D., K.L., Dignard, D., Ziegelbauer, K., Schmidt, A., Gow, N.A., Brown, A.J., Thomas, D.Y., 1996. Signal transduction through homologs of the Ste20p and Ste7p protein kinases can trigger hyphal formation in the pathogenic fungus *Candida albicans*. *Proc. Natl. Acad. Sci. USA* 93, 13217-22.
- Li, M., Martin, S.J., Bruno, V.M., Mitchell, A.P., Davis, D.A., 2004. *Candida albicans* Rim13p, a protease required for Rim101p processing at acidic and alkaline pHs. *Eukaryotic Cell* 3, 741-51.
- Lieber, M., Smith, B., Szakal, A., Nelson-Rees, W., Todaro, G., 1976. A continuous tumor-cell line from a human lung carcinoma with properties of type II alveolar epithelial cells. *Int. J. Cancer* 17, 62-70.
- Liu, H., Köhler, J., Fink, G.R., 1994. Suppression of hyphal formation in *Candida albicans* by mutation of a *STE12* homolog. *Science* 266, 1723-6.
- Lo, H.J., Köhler, J.R., DiDomenico, B., Loebenberg, D., Cacciapuoti, A., Fink, G.R., 1997. Nonfilamentous *C. albicans* mutants are avirulent. *Cell* 90, 939-49.
- Menzel, R., 1989. A microtiter plate-based system for the semiautomated growth and assay of bacterial cells for beta-galactosidase activity. *Anal. Biochem.* 181, 40-50.
- Miller, J., 1972. *Experiments in Molecular Genetics*. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1972.
- Mitchell, L.H., Soll, D.R., 1979. Commitment to germ tube or bud formation during release from

stationary phase in *Candida albicans*. Exp. Cell Res. 120, 167-79.

Murad, A.M., d'Enfert, C., Gaillardin, C., Tournu, H., Tekaia, F., Talibi, D., Marechal, D., Marchais, V., Cottin, J., Brown, A.J., 2001a. Transcript profiling in *Candida albicans* reveals new cellular functions for the transcriptional repressors *CaTup1*, *CaMig1* and *CaNrg1*. Mol. Microbiol. 42, 981–93.

Murad, A.M., Leng, P., Straffon, M., Wishart, J., Macaskill, S., MacCallum, D., Schnell, N., Talibi, D., Marechal, D., Tekaia, F., d'Enfert, C., Gaillardin, C., Odds, F.C., Brown, A.J., 2001b. *NRG1* represses yeast-hypha morphogenesis and hypha-specific gene expression in *Candida albicans*. EMBO J. 20, 4742-52.

Perlroth, J., Choi, B., Spellberg, B., 2007. Nosocomial fungal infections: epidemiology, diagnosis, and treatment. Med. Mycol. 45,321-46.

Pfaller, M.A., Diekema, D.J., 2007. Epidemiology of invasive candidiasis: a persistent public health problem. Clin. Microbiol. Rev. 20, 133-63.

Rocha, C.R., Schröppel, K., Marcus, D., Marcil, A., Dignard, D., Taylor, B.N., Thomas, D.Y., Whiteway, M., Leberer, E., 2001. Signaling through adenylyl cyclase is essential for hyphal growth and virulence in the pathogenic fungus *Candida albicans*. Mol. Biol. Cell 12, 3631-43.

Sasaki, T., Otani, T., Yoshida, K., Unemi, N., Hamada, M., Takeuchi, T., 1997. Discovery of Methyl-2-(2'-hydroxyphenyl)-2-oxazoline-4-carboxylate as a secondary metabolite from *Actinomadura sp.* J. Antibiot. 50, 881-3.

Sato, T., Ueno, Y., Watanabe, T., Mikami, T., Matsumoto, T., 2004. Role of Ca^{2+} /calmodulin signaling pathway on morphological development of *Candida albicans*. Biol. Pharm. Bull. 27, 1281-4.

Saville, S.P., Lazzell, A.L., Bryant, A.P., Fretzen, A., Monreal, A., Solberg, E.O., Monteagudo, C., Lopez-Ribot, J.L., Milne, G.T., 2006. Inhibition of filamentation can be used to treat disseminated candidiasis. Antimicrob. Agents Chemother. 50, 3312-6.

- Schweizer, A., Rupp, S., Taylor, B.N., Röllinghoff, M., Schröppel, K., 2000. The TEA/ATTS transcription factor *CaTec1p* regulates hyphal development and virulence in *Candida albicans*. *Mol. Microbiol.* 38, 435-45.
- Shareck, J., Belhumeur, P., 2011. Modulation of morphogenesis in *Candida albicans* by various small molecules. *Eukaryotic Cell* 10, 1004-12.
- Staab, J.F., Bradway, S.D., Fidel, P.L., Sundstrom, P., 1999. Adhesive and mammalian transglutaminase substrate properties of *Candida albicans* Hwp1. *Science* 283, 1535-8.
- Staab, J.F., Ferrer, C.A., Sundstrom, P., 1996. Developmental expression of a tandemly repeated, proline-and glutamine-rich amino acid motif on hyphal surfaces on *Candida albicans*. *J. Biol. Chem.* 271, 6298-305.
- Stoldt, V.R., Sonneborn, A., Leuker, C.E., Ernst, J.F., 1997. Efg1p, an essential regulator of morphogenesis of the human pathogen *Candida albicans*, is a member of a conserved class of bHLH proteins regulating morphogenetic processes in fungi. *EMBO J.* 16, 1982-91.
- Tortorano, A.M., Peman, J., Bernhardt, H., Klingspor, L., Kibbler, C.C., Faure, O., Biraghi, E., Canton, E., Zimmermann, K., Seaton, S., Grillot, R., ECMM Working Group on Candidaemia, 2004. Epidemiology of candidaemia in Europe: results of 28-month European Confederation of Medical Mycology (ECMM) hospital-based surveillance study. *Eur. J. Clin. Microbiol. Infect. Dis.* 23, 317-22.
- Uhl, M.A., Johnson, A.D., 2001. Development of *Streptococcus thermophilus lacZ* as a reporter gene for *Candida albicans*. *Microbiology* 147, 1189-95.
- Vermes, A., Guchelaar, H.J., Dankert, J., 2000. Flucytosine: a review of its pharmacology, clinical indications, pharmacokinetics, toxicity and drug interactions. *J. Antimicrob. Chemother.* 46, 171-9.
- Wisplinghoff, H., Bischoff, T., Tallent, S.M., Seifert, H., Wenzel, R.P., Edmond, M.B., 2004. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. *Clin. Infect. Dis.* 39, 309-17.

Xu, W., Mitchell, A.P., 2001. Yeast PalA/AIP1/Alix homolog Rim20p associates with a PEST-like region and is required for its proteolytic cleavage. *J. Bacteriol.* 183, 6917-23.

Zhang, J., Chung, T., Oldenburg, K., 1999. A simple statistical parameter for use in evaluation and validation of high throughput screening assays. *J. Biomol. Screen.* 4, 67-73.

Zou, H., Fang, H.M., Zhu, Y., Wang, Y., 2010. *Candida albicans* Cyr1, Cap1 and G-actin form a sensor/effector apparatus for activating cAMP synthesis in hyphal growth. *Mol. Microbiol.* 75, 579-91.

Table 1:

Concentration-dependent effects of hyphal inhibitors from pilot screening on *HWPI* expression, growth of *C. albicans* yeast cells and on human A549 cells. *HWPI*-expression was determined by the β -galactosidase assay after 3 h of exposure to dilution series of the test compounds under hyphae-stimulating conditions. Yeast growth was determined photometrically after 24 h exposure to dilution series of the test compounds under yeast-favouring conditions. Cytotoxicity on A549 cells was evaluated by an MTT-reduction assay after 5 d exposure to dilution series of the test compounds.

Compound	MW	% <i>HWPI</i> expression at 20 mg/l	IC ₅₀ for <i>HWPI</i> expression [μ M]	IC ₅₀ for yeast growth of <i>C. albicans</i> [μ M]	IC ₅₀ for cytotoxicity to A549-cells [μ M]
1	360.0	3 %	< 60	< 60	20
2	292.1	6 %	20	30	90
3	329.3	8 %	50	20	40
4	452.5	7 %	40	60	70
5	422.5	16 %	< 50	< 50	40
6	281.3	7 %	nt	70	70
7	297.3	20 %	< 70	> 70	200
8	241.2	23 %	60	250	r
9	256.2	37 %	< 80	nt	nt
10	274.3	4 %	10	90	40

r: inhibition of vitality 50 % inhibition at the highest concentration tested (250 μ M);

nt: not tested.

Figure Legends

Figure 1: Effect of known inhibitors of hyphal development. A: Production of *o*-nitrophenol by β -galactosidase as a measure of *HWP1*-expression was determined by the reporter assay after three hours of incubation with 100 μ M MDL-12,330A (MDL), or 22 μ M farnesol or the solvent (2 % DMSO). The means and standard deviations were calculated for $n = 48$. B: Microscopical images of the morphology of treated and control cells; scale bars = 20 μ m, recorded on a Keyence BZ-8100E microscope using a 20x plan apochromat objective with a numerical aperture of 0.75, using the manufacturer's software. C: Microscopical images of treated and control cells were analysed by counting the number of hyphae-forming cells. The means and standard deviations were calculated for 6-8 images comprising a total of more than 1000 cells.

Figure 2: Chemical structures of hits from the pilot screening of 720 synthetic compounds from EMC microcollections GmbH that were members of three classes, methyl aryl-oxazoline carboxylates, dihydrobenzo[d]isoxazolones and thiazolo[4,5-*e*]benzoxisoxazoles.

Fig. 1

Fig. 2

Dihydrobenzo[d]isoxazolones

Thiazolo[4,5-c]benzoxazoles

Aryl-oxazoline carboxylates