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

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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 promoter 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*]benzoisoxazoles.

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*.

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). Besides 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 (e.g. 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 morphological switch between the yeast and the hyphae morphology 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 morphology also

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.

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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% CO_2, 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 CO_2-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 *HWP1*. It codes for the adhesin Hwp1p, which was detected in the cell wall of hyphae but never of blastoconidia. Likewise, the *HWP1*-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 *HWP1* 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

C. albicans CAI-4 *HWP1-lacZ* (Hogan et al., 2004) was used in this study. Integration of *lacZ* downstream of the *HWP1*-promoter was confirmed by sequencing products of Uneven PCRs executed according to the method of Chen and Wu (1996) with small changes (see supporting information) at the genome analytics facility of the Helmholtz Centre for Infection Research, Braunschweig, R. Geffers, Working Group GNA, HZI.

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 $OD_{620\,nm}$ was adjusted to 0.05–0.1 as determined using a microtitre plate reader (μQuant, Biotek) in 180 μl sample volume (approx. $4–8 \times 10^6$ 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 was added.

The microtitre plate was incubated at 37 °C for 3 h 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 Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM Mg_2SO_4, 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 (Eq. (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 Eq. (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. The absorption by *o*-nitrophenol was therefore calculated according to Eq. (1).

$$\text{absorption by } o\text{-nitrophenol} = OD_{414\,nm} - c \times OD_{550\,nm} \quad (1)$$