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Wicaltin, a New Protein Toxin Secreted by the Yeast *Williopsis californica* and Its Broad-Spectrum Antimycotic Potential

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Abstract The yeast *Williopsis californica* was shown to secrete a unique broad-spectrum killer toxin (Wicaltin) with antifungal activity against 14 yeast genera, including yeast-like and mycelial forms of the human pathogens *Candida albicans* and *Sporothrix schenkii*. Agar diffusion bioassays indicated that its activity was more pronounced than the antifungal potential of frequently used antimycotics: 0.07 pmol Wicaltin showed the same toxicity as 0.2 pmol miconazole and 29 pmol clotrimazole. Since the toxin's primary target would appear to be the yeast cell wall, Wicaltin may be attractive in combatting clinically relevant yeast and fungal infections.

Key words: Yeast, killer toxin, antifungal

Over the past two decades, the increasing incidence of fungal diseases caused by pathogenic fungi and yeasts, in particular by the opportunistic dimorphic yeast *Candida albicans*, have become a major concern for human health. Immunocompromised patients are highly susceptible to nosocomial infections caused by fungi [5, 6]. Predisposing factors favoring candidiasis include the use of immunosuppressive and cytotoxic therapies or broad-spectrum antibacterial agents, the presence of intravenous catheters, and immunosuppression arising from AIDS, cancer, diabetes, and bone marrow and organ transplantations. Clinical therapy of severe *Candida* infections is often hampered by the limited number of antifungal agents available. In treating fungal infections, synthetic azoles like fluconazole, itraconazole, and ketoconazole are frequently used, as is the polyene amphotericin B. The application of these drugs, especially amphotericin B, is often limited by toxic side effects [1, 2].

Antifungals belonging to the azole family - which are fungistatic rather than fungicidal agents - inhibit lanosterol demethylase, an essential enzyme of plasma membrane ergosterol biosynthesis in yeast and higher fungi [8]. Due to the frequent use of fluconazole and related azoles, an increasing number of cases of resistance have been reported [4, 16].

In the search for novel and more powerful antifungals, yeast and fungal cell wall components represent attractive targets, since these structures are restricted to yeast and higher fungi and are absent from mammals [6, 12]. Yeast cell walls are predominantly composed of a high molecular weight, linear and branched β-1,3-D-glucans, β-1,6-D-glucans, mannoproteins, and (to a much lesser extent) chitin [3]. As some of these wall components act as primary receptors for killer toxins secreted by certain killer strains of different yeasts, the current research focused on the possible use of killer toxins as novel antifungals. Yeast killer toxins are secreted proteins or glycoproteins that kill sensitive yeast strains in a receptor-mediated two-step process, either by disrupting the cytoplasmic membrane function or by blocking the DNA synthesis in the yeast cell nucleus [20]. Although toxin-secreting killer strains have been identified in 13 different yeast genera, the most intensively studied killers are still those belonging to the virally encoded killer system in *Saccharomyces cerevisiae* [reviewed in 13, 20]. Chromosomally encoded as well as plasmid-driven killer toxins have been described in the yeast genera *Candida*, *Cryptococcus*, *Hansenula*, *Kluyveromyces*, *Pichia*, *Torulopsis*, and *Williopsis* [10, 11, 13, 14]. The toxin-secreting killer strains in the yeast *Williopsis* show a wide spectrum of intergeneric killing activity, and two low molecular weight killer toxins secreted by *W. mrakii* have already been purified and characterized: the K-500 toxin (5.0 kDa) and the 10.7 kDa HM-1 toxin [7, 9, 21].

This study describes a novel killer strain of *W. californica* strain DSM 12865, whose secreted toxin (Wicaltin) exhibits toxicity against a broad spectrum of yeasts. Wicaltin exhibits optimal killing at pH 4.7 and 20°C. Since toxin production is blocked in yeast cells treated with sublethal concentrations

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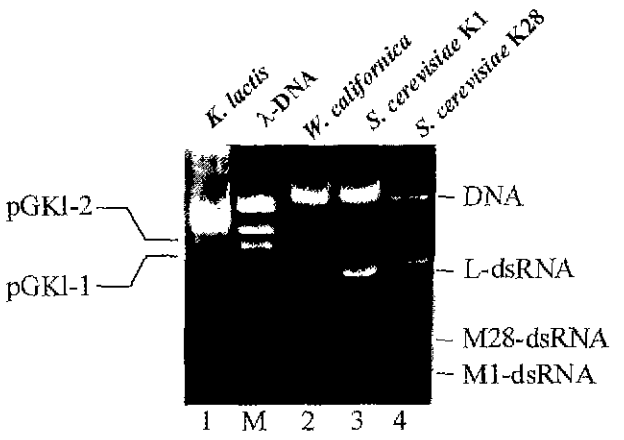

Fig. 1. Neither dsRNA genomes nor dsDNA plasmids are detectable in *W. californica* killer cells.
The viral L- and M-dsRNA genomes isolated from the *S. cerevisiae* killer strains *MS300c* and *K7* (*lanes 4 and 3, respectively*), the linear dsDNA plasmids pGKl-1 and pGKl-2 from the *Kluyveromyces lactis* killer strain IFO1267 (lane 1), and a nucleic acid preparation of *W. californica* killer cells (lane 2) were subjected to electrophoresis on a 0.8% agarose gel and subsequently visualized by ethidium bromide staining. The presence and positions of the chromosomal yeast DNA, the viral L- and M-dsRNA genomes, as well as the dsDNA plasmids pGKl-1 and pGKl-2 are indicated (M represents an *Hin*dIII-digested λDNA marker). Nucleic acid isolation and electrophoretic analysis was performed as previously described [17, 18]

of cycloheximide, and the isolated toxin is rapidly inactivated by treatment with either pronase E, proteinase K, or trypsin, Wicaltin belongs to the family of secreted yeast protein (killer) toxins. Furthermore, since electrophoretic analysis of *W. californica* cell extracts indicated that neither cytoplasmic persisting double-stranded (ds)RNA viruses nor toxin-coding dsDNA plasmids were detectable in *W. californica* killer cells (Fig. 1), Wicaltin would appear to be encoded by a chromosomal yeast gene. In order to isolate Wicaltin in a preparative scale, the killer strain was grown for 5 days at 20°C under gentle shaking in a 10 l synthetic BAVC-medium (pH 4.7). The yeast culture medium used in this study (BAVC) represents the originally described B-medium [15], except that the concentrations of vitamins and amino acids were doubled. Cell-free culture supernatants of the killer strain were concentrated 200-fold by ultrafiltration (Easy-Flow, Sartorius) using membranes with a molecular cut-off of 10 kDa. The resulting toxin concentrate (50 ml) was dialyzed against a 5 mM McIlvaine buffer (pH 4.7) and single-step Wicaltin purification was achieved by cation-exchange chromatography on a Bio-Scale S column (Bio-Rad, Germany). In contrast to the low molecular weight protein toxins HM-1 (10.7 kDa) and HYI (9.5 kDa) secreted by killer strains of *W. mrakii* and *W. saturnus* [9, 21], SDS-PAGE of the purified *W. californica* toxin indicated that Wicaltin is a 34 kDa protein toxin whose activity was previously determined in a standard agar diffusion well test bioassay [19]. As summarized in Table 1

Table 1. Broad-spectrum toxicity of Wicaltin secreted by the yeast *W. californica* DSM 12865 against pathogenic and non-pathogenic yeasts.

Yeast species	Strain number/source	Wicaltin sensitivity [mm: growth inhibition]
Candida albicans	Clinical isolate	17
C. glabrata	Clinical isolate	17
C. tropicalis	Clinical isolate	11
Debaryomyces hansenii	223	16
Kluyveromyces lactis	CBS 2359/152	22
Metschnikowia pulcherrima	K/3IB6	8
Pichia anomala	245	17
P. jadinii	251	6
Saccharomyces cerevisiae	192.2d	30
Sporothrix schenkii	1129	11
Torulaspora delbrueckii	208	18
T. pretoriensis	186	10
Yarrowia lipolytica	271	8
Zygosaccharomyces bailii	DSM 12864	23

The toxin sensitivity of the indicated yeast genera was determined using a standard agar diffusion well plate assay on a methylene blue agar (pH 4.7) against Wicaltin at a concentration of 20 μg ml^{-1}.

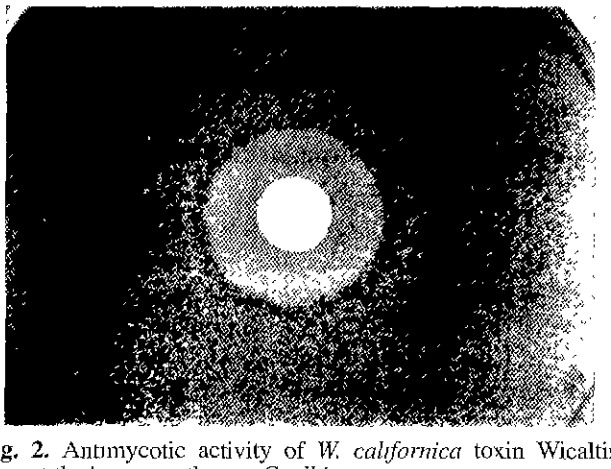

Fig. 2. Antimycotic activity of *W. californica* toxin Wicaltin against the human pathogen *C. albicans*.
Aliquots (2×10^5 cells) of a pathogenic strain of *C. albicans* isolated from bronchi aspirates were embedded in a liquified (48°C) methylene blue agar (pH 4.7) and 100 μl of purified Wicaltin were pipetted into wells (9 mm in diameter) cut into the agar. After incubating the plate for 3 days at 20°C, a clear zone of growth inhibition around the wells indicated toxin activity.

and illustrated in Fig. 2, Wicaltin showed a broad-spectrum activity against 14 different yeast genera, including 17 clinical isolates of human pathogenic strains of *C. albicans* and one clinical isolate from *C. glabrata*, *C. tropicalis*, and *Sporothrix schenkii*. All clinically relevant strains of *C. albicans* were isolated from either gastric juice, sputum, throat swabs, or bronchi or tracheal aspirates of the patients suffering from acute and severe mycoses. *C. albicans* was not only sensitive when growing in a yeast-like form, but

also similarly pronounced in a mycelial state after germ tube induction through cultivation at 37°C in the presence of fetal calf serum (data not shown).

In order to determine the toxin sensitivity of the yeast *Sporothrix schenkii* and study the effect of the osmotic stabilizing sugar alcohol sorbitol on the survival rate of toxin-treated cells, 1.0×10^6 cells of *S. schenkii* were first treated with EDTA (5 mM) and DTT (5 mM) in a McIlvaine buffer (pH 4.7, 100 mM) for 1 h at 20°C, and thereafter incubated in a YEPD medium at 20°C in the presence or absence of Wicaltin (20 μg ml^{-1}) and/or sorbitol (1.2 M). As shown in Fig. 3, the yeast cell viability decreased significantly in the presence of Wicaltin, and after 52 h the cell survival represented only 0.2% of the cell numbers in the toxin-free control. Interestingly, the addition of sorbitol had a protective effect on the toxin-treated cells resulting in a 10-fold increase in cell survival. This result indicates that the primary *in vivo* target of Wicaltin would appear to be the yeast cell wall, since cell wall damage in yeast and higher fungi is known to affect osmotic cell stability and dramatically decreases cell viability in non-osmotically stabilized culture media. However, even sorbitol-stabilized yeast cells were effectively killed by Wicaltin (Fig. 3), thereby indicating that the toxin's mode of action must be different from that of classical cell wall digesting enzymes like glucanases, mannanases, or even the well-known β-1,3-D-glucan hydrolyzing Zymolyase. Therefore, it was then investigated whether Wicaltin exhibits toxicity against regenerating yeast cell spheroplasts. In order to address this experimentally, freshly isolated spheroplasts of the human pathogenic yeast *C. albicans* were embedded in a liquified regeneration agar [19] and tested against a purified toxin preparation using a standard agar diffusion well plate assay [19]. Interestingly, just like the intact cells,

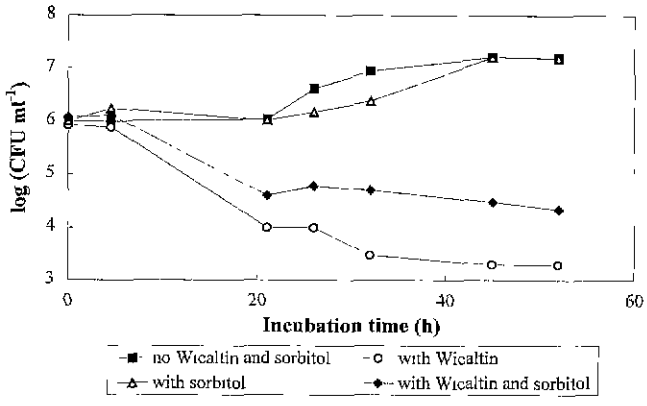

Fig. 3. Influence of Wicaltin on cell viability of *S. schenkii* with and without the osmotic stabilizing sugar alcohol sorbitol.
The sensitive yeast was incubated at 20°C in a YEPD medium (pH 4.7) in the presence or absence of Wicaltin (20 μg ml^{-1}) and/or sorbitol (1.2 M) and assayed for cell survival by plating aliquots onto a YEPD regeneration agar (0.6 M KCl, pH 7.0). The cell numbers were determined after 3 days of incubation at 30°C

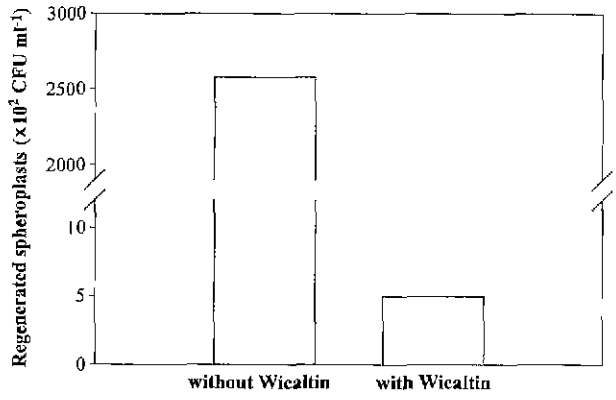

Fig. 4. Wicaltin dramatically decreased survival of regenerating yeast cell spheroplasts.
Spheroplasts of the yeast *C. albicans* (3×10^5 CFU ml^{-1}) were treated with 20 μg ml^{-1} Wicaltin for 6 h at 20°C, washed, and subsequently embedded in a liquified regeneration agar (pH 7.0). After 6 days of incubation at 30°C, the yeast cell survival was determined by colony counting. In a toxin-free control, the yeast cell spheroplasts were resuspended in a McIlvaine buffer (0.1 M, pH 4.7) and treated in the same way

Candida spheroplasts turned out to be highly sensitive to the toxin. To determine the effect of Wicaltin on regenerating yeast cell spheroplasts, Zymolyase-treated cells of the same yeast (*C. albicans*) were incubated for 6 h at 20°C in the presence or absence of 20 μg ml^{-1} Wicaltin. Thereafter, the spheroplasts were washed, diluted in a spheroplast regeneration medium (pH 4.7), and embedded in a liquified regeneration agar (pH 7.0). After incubating the plates for 5 days at 30°C, the cell survival was determined by colony counting. As shown in Fig. 4, Wicaltin dramatically decreased the cell survival, and the spheroplast regeneration rate in the Wicaltin-treated cells was only 0.16%, in contrast to 83.5% in the toxin-free control. These results strongly suggest that Wicaltin is not just a simple cell-wall-hydrolyzing protein, but rather a highly effective toxin that somehow inhibits cell wall regeneration (perhaps at the level of cell wall β-1,3-D-glucan synthesis). Because of its broad-spectrum killing activity, Wicaltin is particularly interesting as a novel antimycotic protein toxin. Accordingly, agar diffusion bioassays were used to compare the antimycotic potential of Wicaltin with the fungistatic activity of well-known and frequently used topical antifungals such as clotrimazole and miconazole. As illustrated in Fig. 5, Wicaltin at a molar concentration of 0.07 pmol showed the same anti-*Candida* activity as 29 pmol clotrimazole or 0.2 pmol miconazole. Thus, on a molar basis, Wicaltin is even more effective than other frequently used antifungals. Together with the high prevalence of toxin-sensitivity among pathogenic yeasts, this data indicates that Wicaltin is an attractive candidate for use as an antifungal, preferably in treating topical mycoses. Therefore, the future experiments will focus on (i) identifying the toxin's primary target, (ii) cloning the corresponding Wicaltin-coding gene, and (iii) heterologous Wicaltin

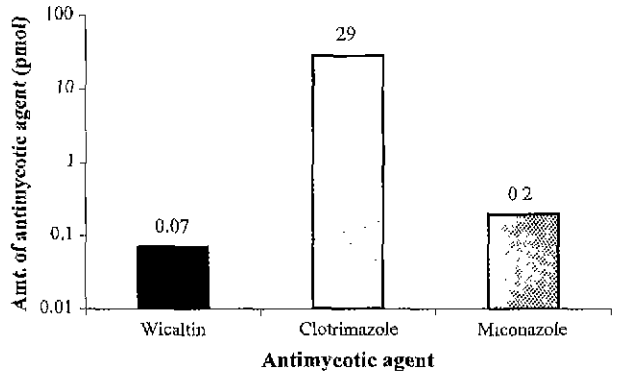

Fig. 5. Comparative anti-*Candida* activity of purified Wicaltin and the frequently used antifungals clotrimazole and miconazole, determined using a standard agar diffusion bioassay on methylene blue agar (MBA, pH 4.7).
On MBA plates, the indicated molar amount of each antifungal caused a cell-free zone of growth inhibition of 12 mm.

expression in Baker's and in fission yeast in order to isolate the toxin on a preparative scale that will make it amenable to further pharmacological studies and even clinical trials.

Acknowledgments

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