

# Inhibitory Effect of Terbinafine on Reactive Oxygen Species (ROS) Generation by *Candida albicans*

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Application Note 106

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- Intracellular ROS detection by luminescence
- LOQ of  $>10^7$  cells in 200  $\mu$ L
- Time- and dose-dependent inhibition of ROS generation

## Introduction

*Candida albicans* is one of the most important opportunistic fungal pathogens. The yeast is able to cause candidiasis, a disease that can vary from superficial mucosal to life-threatening systemic disorders. Several virulence factors of *C. albicans* are known including the dimorphism, which is the ability to grow in either a yeast-like (blastoconidial) or a mycelial (hyphal) form in response to different environmental factors.<sup>1</sup> The argument that hyphae represent an advantage in tissue invasion seems obvious and widely accepted, but definitive experimental evidence is lacking.<sup>2</sup>

Reactive oxygen species (ROS) receive increasing attention as short acting compounds involved in inflammation and tissue damage. ROS can react with polyunsaturated fatty acids in cellular membranes, nucleotides, and sulphhydryl bonds in proteins<sup>3</sup>, and have been related to tissue injury in yeast infections.<sup>4</sup>

In *C. albicans*, mitochondria are capable of generating and releasing extracellular ROS.<sup>5</sup> In our previous work, we showed that ROS generation by *C. albicans* is dependent on morphogenesis and is highest in hyphal form cells.<sup>6</sup> Thus, ROS generation may play a major role in tissue invasion and infection.

Terbinafine, a synthetic allylamine antifungal agent, inhibits ergosterol biosynthesis via squalene epoxidase. When exposed to terbinafine, fungi accumulate squalene while becoming deficient in ergosterol, an essential component of fungal cell membranes. The inhibition of squalene epoxidase leads to fungistatic and fungicidal action.<sup>7</sup> There is also evidence that terbinafine, at therapeutic concentrations, can be considered a free radical interceptor *in vitro* and could exert an anti-inflammatory activity *in vivo*.<sup>8</sup> The substance is capable of interacting with cell membranes and inhibiting the generation of reactive oxygen species.<sup>9,10</sup>

In this study, the effect of terbinafine on ROS generation was analyzed. We investigated whether terbinafine influences the oxidant properties of this pathogen.

## Materials

*C. albicans* strain 3153A was pre-cultured in yeast extract peptone glucose (YEPG) medium 21 h at 25°C to obtain cells in the yeast phase (blastoconidiae). Samples at the concentration of  $1 \times 10^6$  cells/mL to

$1 \times 10^9$  cells/mL were prepared in saline and YEPG medium. *Candida* cells at a concentration of  $1 \times 10^8$  cells/mL were incubated in saline with terbinafine at concentrations of 1  $\mu$ g/mL, 10  $\mu$ g/mL and 100  $\mu$ g/mL dissolved in DMSO. Incubation was performed for 10 min and 60 min at 25°C in a shaking incubator. The duration was restricted to exclude effects of terbinafine on cell number and growth.

ROS measurements were carried out by means of lucigenin amplified chemiluminescence (CL) as described.<sup>11</sup> Lucigenin solution was prepared in PBS at a concentration of 0.1 mmol/L. A 200  $\mu$ L of fungal cell suspension was measured in the tube luminometer LB 953 (Berthold, Germany) or in the LUMIstar microplate reader (BMG LABTECH GmbH, Germany). Chemiluminescent signals were recorded using the slow kinetic method for yeast cells at different cell numbers and for  $1 \times 10^8$  cells/mL incubated with terbinafine. The calculation was performed using the average counts per minute (cpm) or RLU over measurement periods of 20 min.

All data was expressed as means  $\pm$  standard deviation. Each test was replicated. Data was analyzed using Student's t-test for paired data.  $P < 0.05$  was considered statistically significant.

## Results and Discussion

Generation of reactive oxygen species was detectable in *C. albicans* blastoconidiae. The results were dependent on yeast cell number and media used for measurement. Chemiluminescent signals could be found at concentrations  $>1 \times 10^6$  cells/mL of *C. albicans* blastoconidiae. The maximum was measured at concentrations  $>1 \times 10^8$  cells/mL (Fig. 1). At higher concentrations ROS generation was inhibited in both media, which is very likely to be a protective mechanism.

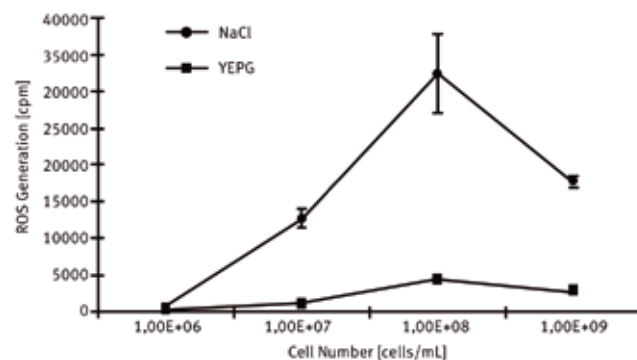
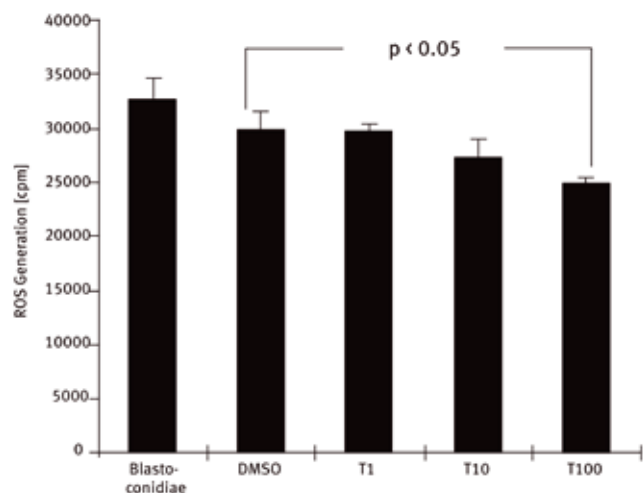


Fig. 1: Effect of increasing numbers of *Candida albicans* blastoconidiae on generation of ROS, measured by lucigenin amplified chemiluminescence in NaCl solution and YEPG medium.

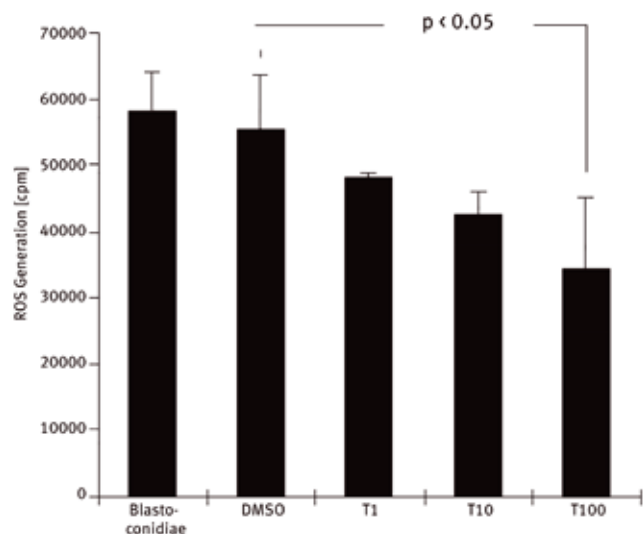
The media used for measurements had strong effects on total amounts of reactive oxygen species. Measurements in saline revealed the highest values, which could be explained by the lack of nutrients in this solution and resulting stress within the cells. Furthermore, YEPG medium presumably possesses light quenching effects in chemiluminescent measurements.<sup>6,12</sup> Due to these results, further experiments were carried out with blastoconidiae at a cell number of  $1 \times 10^8$  cells/mL in saline.

After 10 min of incubation with terbinafine, a dose-dependent inhibition of ROS generation was already seen (Fig. 2). DMSO itself, as the soluble reagent, revealed antioxidant properties. However, the inhibition of ROS generation was considerably dependent on the used terbinafine concentration. At a terbinafine concentration of 1 µg/mL (T1) the inhibition corresponded to 9.4 % and at 100 µg/mL (T100) to 24 %.



**Fig. 2:** Dose-dependent inhibition of ROS generation by *Candida albicans* after 10 min incubation with different concentrations of terbinafine. Blastoconiae cell number:  $1 \times 10^8$  cells/mL in saline. All other samples contain yeast cells and additionally DMSO or terbinafine.  
DMSO: + 1 % DMSO,  
T1: + 1 µg/mL terbinafine,  
T10: + 10 µg/mL terbinafine,  
T100: + 100 µg/mL terbinafine.

The inhibition was significant for the comparison of the control incubated with DMSO and the highest chosen terbinafine concentration of 100 µg/mL (T100) ( $p < 0.05$ ). After 60 min of incubation with terbinafine the inhibition was amplified (Fig. 3). The final inhibitory effect was 18% for 1 µg/mL terbinafine (T1) and 41.1% for 100 µg/mL terbinafine (T100). The effect was significant for terbinafine concentrations of 10 µg/mL (T10) and 100 µg/mL (T100) ( $p < 0.05$ ). The dose of 10 µg/mL (1%) corresponds to the dose applied for topical preparations.<sup>7</sup>



**Fig. 3:** Dose-dependent inhibition of ROS generation by *Candida albicans* after 60 min incubation with different concentrations of terbinafine. Blastoconiae cell number:  $1 \times 10^8$  cells/mL in saline. All other samples contain yeast cells and additionally DMSO or terbinafine.  
DMSO: + 1 % DMSO,  
T1: + 1 µg/mL terbinafine,  
T10: + 10 µg/mL terbinafine,  
T100: + 100 µg/mL terbinafine.

These results reveal a terbinafine time- and dose-dependent inhibition of ROS generation by *C. albicans*. Due to the time-dependency, it is likely that terbinafine not only has radical scavenging properties, but also interacts with the pathway that generates reactive oxygen species. In summary, terbinafine reduces the ability of *C. albicans* to generate reactive oxygen species. Besides the known influence on ergosterol biosynthesis, this mechanism may contribute to antifungal action and improvement of the inflammatory situation within the host. Further investigations must prove whether terbinafine inhibits generation of reactive oxygen species as well in the hyphal phase, which is associated with markedly increased ROS generation.<sup>6</sup>

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