



## Photodynamic therapy of oral *Candida* infection in a mouse model



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### ABSTRACT

Species of the fungal genus *Candida*, can cause oral candidiasis especially in immunosuppressed patients. Many studies have investigated the use of photodynamic therapy (PDT) to kill fungi *in vitro*, but this approach has seldom been reported in animal models of infection. This study investigated the effects of PDT on *Candida albicans* as biofilms grown *in vitro* and also in an immunosuppressed mouse model of oral candidiasis infection. We used a luciferase-expressing strain that allowed non-invasive monitoring of the infection by bioluminescence imaging. The phenothiazinium salts, methylene blue (MB) and new methylene blue (NMB) were used as photosensitizers (PS), combined or not with potassium iodide (KI), and red laser (660 nm) at four different light doses (10 J, 20 J, 40 J and 60 J). The best *in vitro* log reduction of CFU/ml on biofilm grown cells was: MB plus KI with 40 J (2.31 log;  $p < 0.001$ ); and NMB without KI with 60 J (1.77 log;  $p < 0.001$ ). These conditions were chosen for treating the *in vivo* model of oral *Candida* infection. After 5 days of treatment the disease was practically eradicated, especially using MB plus KI with 40 J. This study suggests that KI can potentiate PDT of fungal infection using MB (but not NMB) and could be a promising new approach for the treatment of oral candidiasis.

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### 1. Introduction

A leading cause of opportunistic infections are fungal species belonging to the *Candida* genus, with *Candida albicans* ranking as the most prevalent causative agent of candidiasis and candidemia around the world [1–6]. This trend has been observed over the past decade and is still the case, even in developed countries such as the United States, Denmark, Norway, and Finland [3]. *C. albicans* is a human commensal organism, and can colonize the skin, mucosal surfaces, gastrointestinal tract and the female genitourinary tract [7]. One important factor that contributes to the pathogenesis of candidiasis is biofilm formation, as *C. albicans* has the ability to form biofilms on both inert and biological surfaces [8]. These biofilms are typically surrounded by an extracellular polymeric substance [9] which effectively shelters the fungal cells against adverse environmental conditions, including host defense mechanisms and the action of antifungal drugs [10]. Research and development of new antifungal agents is complicated by the paucity of selective microbial targets, since fungi are also eukaryotic cells [11]. In addition, drug resistance of *C. albicans* against antifungals such as azoles and echinocandins represents an increasing problem [12].

Therefore, novel antifungal and antibiofilm drugs against these unmanageable infections are urgently needed [10,11]. Photodynamic therapy (PDT) has emerged as a promising modality due to its effectiveness against a broad range of species of microorganisms regardless of drug resistance [13]. In this approach, a photosensitizing agent or dye, which is activated by a light source at a specific wavelength in the presence of oxygen, resulting in the production of reactive oxygen species (ROS) and free radicals, is administered into the infected area. These photogenerated ROS disrupt the *Candida* cytoplasmic membrane and cause an increase in cellular permeability and subsequent damage to intracellular targets. It has been suggested that this oxidative stress might compromise virulence factors of the microorganism, such as the capacity to adhere to host epithelial cells, production of proteinases, reduction of biofilms and formation of germ tubes [14–20].

Mice have been used as models to study oral *C. albicans* infections and offer some advantages over a rat model in that mice do not harbor *Candida* spp. in their buccal cavity and therefore do not elicit an adaptive immune response against this yeast [21,22]. Optimally, the detection of light from small animals containing bioluminescent cells can be achieved using a charge-coupled device (CCD) based imaging system [23] allowing real-time non-invasive monitoring of the infection.

We have found no studies in the literature that monitored oral *C. albicans* infections in mice by bioluminescence imaging after use of photodynamic therapy, which could be a therapeutic option for the treatment of oral candidiasis. PDT could be a sole treatment option, or could be used as a coadjuvant to antifungal chemotherapy, improving

Abbreviations: PDT, photodynamic therapy; MB, methylene blue; NMB, new methylene blue; PS, photosensitizers; KI, potassium iodide.

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the treatment of oral infections by increasing therapeutic efficacy and by reducing costs.

For these reasons the objective of the present study was to monitor oral candidiasis produced by a luciferase-expressing *C. albicans* strain (CEC 749) in immunosuppressed mice using bioluminescence imaging before and after PDT using four different doses of red light (10 J, 20 J, 40 J and 60 J) with methylene blue or new methylene blue combined or not with potassium iodide to possibly potentiate the PDT effect. We have recently reported that addition of KI could potentiate antimicrobial PDT *in vitro* and *in vivo* mediated by MB [24], and also PDT mediated by cationic fullerenes in a mouse model of a wound abrasion infected by *Acinetobacter baumannii* [25].

## 2. Materials and Methods

### 2.1. Ethics Committee

All animal procedures in this study were approved by the Subcommittee on Research Animal Care (IACUC) of the Massachusetts General Hospital under the protocol (2015N000099) and were in accordance with the guidelines of the National Institutes of Health (NIH).

### 2.2. Cells and Culture Conditions

A luciferase-expressing *C. albicans* strain (CEC 749) was used in this study [26]. The luciferase reporter was constructed by fusing a synthetic, codon-optimized version of the *Gaussia princeps* luciferase gene (gLUC59) to *C. albicans* PGA59, which encodes a glycosylphosphatidylinositol linked cell wall protein. Luciferase expressed from this PGA59-gLUC fusion was localized at the *C. albicans* cell surface [26], where it can easily come into contact with its substrate coelenterazine (Gold Biotechnology, Inc., Charlestown, MA, USA) in intact cells. This system allows bioluminescence imaging of *C. albicans* infections after simple addition of coelenterazine solution to the surface of the infected tissue [26]. This approach also allows the detection of luciferase in both hyphae and yeast cells [26,27], given the central importance of the yeast-hyphal transition in *C. albicans* virulence [28].

*C. albicans* was routinely grown at 30 °C on yeast extract-peptone-dextrose (YPD) agar and subcultured in YPD medium. The inoculum was prepared by growing the *C. albicans* cells in 3 ml YPD broth overnight at 30 °C with shaking at 130 rpm in a C24 incubator/shaker (New Brunswick Scientific, Edison, NJ). The broths were then centrifuged at 12,000 rpm for 3 min (centrifuge 5417 C; Eppendorf, Hamburg, Germany) and resuspended in 0.5 ml of phosphate-buffered saline (PBS) or YPD. The concentrations were then adjusted to the appropriate values for the experiment by measuring the optical density at 570 nm (OD570) of the *C. albicans* suspensions, using an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific). An OD570 of 0.65 correspond to a fungal concentration of 10<sup>7</sup> CFU/ml in PBS (*in vivo* test) or in YPD media (*in vitro* test).

### 2.3. Photosensitizers and Light Source

The phenothiazinium salts methylene blue (MB) (M9140-25G; Sigma), and new methylene blue (NMB) (556416-1G; Sigma) with or without potassium iodide (KI) were used as PSs in the study. The dye contents of MB and NMB were approximately 85% and 70%, respectively. These photosensitizers were selected by a preliminary study based on the criterion that the concentration produced an effective PDT effect with minimal dark toxicity [29]. Stock PS solutions (10 mM) were prepared by dissolution of the PS powders in sterilized water and were stored in the dark at 4 °C for no longer than 14 days.

The light source used in this study was emitted by a diode laser (Arroyo Instruments, LLC, San Luis Obispo, CA, USA) at a wavelength of 660 nm, at a power of 100 mW, delivering 10 J, or 20 J, or 40 J, or 60 J.

All parameters are given in Table 1. For *in vitro* experiments, the laser beam was expanded by a lens (model LM2-B) to form a spot diameter 4.7 cm delivered without contact with the plates (distance of 7 cm). For *in vivo* experiments, the same laser beam was delivered using an optic fiber without a lens, without contact with mouse tongue (distance of 5 cm), covering a spot area of 0.38 cm<sup>2</sup>.

Both the best sets of conditions from *in vitro* experiments using PDT with NMB (NMB 60 J) and MB (MB/KI 40 J) were chosen to be applied on the *in vivo* model of oral candidiasis. It is important to highlight that the light parameters were kept the same from the *in vitro* experiments (wavelength, total power and irradiation time). The optical power reaching the plates (*in vitro*) and the mouse tongue (*in vivo*) was measured using an optical energy meter PM100D Thorlabs® fitted with a sensor S310C (3.14 cm<sup>2</sup>).

### 2.4. In vitro Photodynamic Therapy

In order to grow the biofilms a 250 µl aliquot of the suspension (10<sup>7</sup> CFU/ml in YPD) was pipetted into each well of a 96-well flat-bottom microtiter plate. The plate was incubated for 1.5 h at 30 °C in a shaker at 75 rpm for the initial adhesion phase. After this period, the wells were washed with 250 µl of PBS to remove loosely adhered cells. A 250 µl aliquot of YPD was then pipetted into each washed well, and the plate was incubated at 30 °C in a shaker at 75 rpm for 48 h. The broth was changed after 24 h. The plate with biofilms formed by *C. albicans* was then washed with 250 µl of PBS to remove loosely adherent cells.

The biofilm formed by each strain was immersed in 100 µl of a solution of each photosensitizer (PS) or PBS for 10 min (pre-irradiation time). Subsequently, the suspended plate was irradiated according to the groups:

- P – L –: received PBS in absence of light. (n = 6).
- P – L +: the effect of the light source only with different doses 10, 20, 40 and 60 J. (n = 6 for each dose).
- P + L –: the effect of the PS only [MB (100 µM) or NMB (100 µM) and these PSs combined with KI (100 mM)]. (n = 6 for each PS).
- P + L +: treatment with each PS [MB (100 µM) or NMB (100 µM) and these PSs combined with KI (100 mM)] and each dose of light (10, 20, 40 and 60 J). (n = 6 for each PS and dose of light).

After this, each well received 100 µl of PBS and the biofilm was disrupted by homogenizing for 1 min using an ultrasonic homogenizer. The suspensions in the wells were considered to be a dilution factor of 10<sup>-1</sup>. 5 serial dilutions were then made using each original 10<sup>-1</sup> dilution, and aliquots of 10 µl were seeded onto YPD agar plates [30] that were then incubated at 30 °C for 24 h. After the incubation period, the CFU/ml values of each plate were determined.

**Table 1**  
Parameters of light for photodynamic therapy (PDT).

<i>In vitro</i>	<i>In vivo</i>
Wavelength (nm): 660	Wavelength (nm): 660
Pulse frequency: continuous	Pulse frequency: continuous
Laser power (mW): 100	Laser power (mW): 100
Irradiation time (sec): 100/200/400/600	Irradiation time (sec): 400/600
Total energy (J): 10/20/40/60	Total energy (J): 40/60
Energy density (J/cm <sup>2</sup> ): 3.18/6.36/12.73/19.10	Energy density (J/cm <sup>2</sup> ): 105.26/157.89
Power density (mW/cm <sup>2</sup> ): 31.84	Power density (mW/cm <sup>2</sup> ): 263.15
Application mode: without contact	Application mode: without contact

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