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# Effect of photodynamic antimicrobial chemotherapy on *Candida albicans* in the presence of glucose

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ABSTRACT

*Background:* *Candida albicans* is an opportunistic commensal microorganism, often associated with severe infections in immunosuppressed individuals. *C. albicans* has hexose transporters that may favor the intracellular accumulation of photosensitizer (PS). the aims of this study were to investigate the influence of glucose load on photodynamic antimicrobial chemotherapy (PACT); and the role that membrane transport system plays on this therapy in the presence of glucose.

*Material and methods:* Strains of *C. albicans* were selected: ATCC 10231, YEM 12, YEM 13, YEM 14 and YEM 15. All strains were grown aerobically on Sabouraud agar and incubated at 30 °C for 24 h. The strains were treated with and without glucose, and divided into Control (no treatment), LED light (660 nm, 166 mW/cm$^2$), Photosensitizer (100 μM methylene blue) and PACT at 1, 3 and 6 min of irradiation groups. The colony forming units were counted and data submitted to statistical analysis (ANOVA) and Tukey's test. The concentration of methylene blue (MB) outside the yeast was measured by fluorescence spectroscopy.

*Results:* PACT inactivate *C. albicans* and the presence of glucose did not affect the killing effect for most strains. Only YEM12 was partially affected by its presence. Regarding efflux systems, ABC overexpressing strain showed a protective effect on the yeast cells. We observed that yeast with overexpression of major facilitator superfamily (MFS) membrane pore tended to accumulate more MB in its cytoplasm, whereas strains that overexpressed ABC pumps (ATP-binding cassette transporters) tended to decrease MB uptake and survive the photodynamic challenge.

*Conclusion:* Presence of glucose showed a small effect on PACT . The accumulation of MB on yeast induces more photodynamic inactivation; however, the photodynamic efficacy depends on the type and characteristics of the microbial strain.

## 1. Introduction

*Candida albicans* is a fungus that can be found in the normal gastrointestinal and genitourinary tract of approximately 80% of the individuals. However, under certain conditions, it becomes a pathogen, responsible for 15% of cases of septicemia [1]. Due to its opportunistic nature, it has frequently been associated with severe infections in immunosuppressed individuals [1,2].

Azole antifungals, especially fluconazole, are a commonly prescribed drug for treatment of *Candida* infections [3]. Nevertheless, the incidence of inherent and acquired azole resistance has been reported due to several mechanisms, including efflux systems. The efflux of drugs via transport proteins that reduce intracellular drug accumulation is found in several *Candida* species. In *C. albicans* there are two main classes of efflux proteins, the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) class of transporters. ABC transport proteins depend on the hydrolysis of ATP for energy and two transporters are linked to fluconazole resistance, the *Candida* drug resistance 1 (Cdr1p) and *Candida* drug resistance 2 (Cdr2p). MFS are powered by electrochemical proton-motive force and only one protein, Mdr1p, is

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related to fluconazole resistance [4].

Confronted with microbial resistance, there exists great interest in the development of other forms of treatment, and photodynamic antimicrobial chemotherapy (PACT) has emerged as a promising strategy [5,6]. This therapy is based on the interaction of a non-toxic photosensitizer with a suitable wavelength of light. This combination, in the presence of oxygen, leads to the formation of reactive oxygen species, which stimulate a cascade of biological events that induce the death of microorganisms [6]. Studies have shown that *C. albicans* presents sensitivity to PACT [7–10]. However, photosensitizer uptake in fungal cells can be affected by plasma membrane efflux systems, which could interrupt cellular inactivation by PACT [5] The response of *Candida albicans* to the presence of glucose is still poorly understood. It is assumed that glucose sensors generate an intracellular signal by activating the hexose transporters [11]. Thus, it becomes necessary to investigate whether the activation of the carriers opens a gateway for the PS. The study of superexpressive strains of active transport (ABC) and passive transport (MFS) may open the door to a better understanding of the metabolism of *C. albicans* by considering the efflux and influx of MB after the cells have been exposed to glucose. For this reason, the aims of this study were to investigate the influence of glucose load on PACT; and also, the role that membrane transport system plays on this therapy in the presence of glucose.

## 2. Materials and methods

### 2.1. Strains and culture conditions

The *C. albicans* strains used in this study are listed in Table 1. Strains were grown in Sabouraud dextrose agar for 24 h at 30 °C. YEM strains were provided by Dr. Olga Lomovskaya (MPEX Pharmaceuticals, San Diego, CA, USA), Professor Dominique Sanglard (Institute of Microbiology, University Hospital Lausanne, Lausanne, Switzerland) Dr. George Tegos and Dr. Michael Hamblin and they were stored at -80 °C at the Center for Lasers and Applications (IPEN-CNEN/SP).

Yeast inoculum was prepared in phosphate buffered saline (PBS) from 24-h cultures. Turbidity of cell suspensions was measured in a spectrophotometer at 540 nm in order to obtain suspensions with an optical density of 0.14 ($1 \times 10^6$ CFU/mL) [15].

### 2.2. Photosensitizer and glucose preparation

A stock solution of 10 mM methylene blue (MB, Sigma-Aldrich MO, USA) was prepared in distilled water and the solution was filtered onto a sterile membrane (0.22 µm, Milipore, São Paulo, Brazil). A 50 mM glucose (Sigma-Aldrich MO, USA) solution was prepared by diluting the glucose powder in PBS and filtering onto a sterile membrane (0.22 µm, Milipore, São Paulo, Brazil).

### 2.3. PACT studies

*C. albicans* strains were tested with different groups: control, no treatment at all; light irradiation and no photosensitizer; (0 irradiation, where *C. albicans* strains were incubated with MB in dark conditions; and PDT groups at 1, 3 and 6 min. of irradiation. In addition, these groups were tested with or without glucose. In groups without glucose, the *C. albicans* cells were incubated with 100 µM MB for 10 min, at room temperature and in the dark [7]. In groups with glucose, the strains were pre-treated with glucose (50 mM glucose for 120 min at room temperature) before receiving MB. However, in the PACT glucose groups, cells were first incubated with glucose and then, after 110 min, MB was added to the suspension.

**Table 1**
Description of *C. albicans* strains used in this study.

| Strains | Characteristics |
|---|---|
| ATCC 10231 | Reference strain isolated from bronchomycosis |
| YEM 12 | Wild type parent of YEM 13 [12] |
| YEM 13 | Overexpressing *MDR1* [12] |
| YEM 14 | Wild type parent of YEM 15 [13,14] |
| YEM 15 | Overexpressing *CDR1*, *CDR2* [13,14] |

Irradiation was performed with a LED device emitting wavelength at $\lambda = 660$ nm at a radiant power of 473 mW. The samples were irradiated from the top of a 24-well microtitre plate with a radiant exposure rate of I = 165 mW/cm$^2$ and radiant exposure of 10, 30 and 60 J/cm$^2$. During irradiation, aliquots were collected and they were serially diluted 10-fold in PBS to give dilutions from $10^{-1}$ to $10^{-4}$ times the original concentrations. Ten-mL aliquots were streaked horizontally on Sabouraud agar plates and incubated at 35 °C for 20–24 h [16]. Colony form units (CFUs) were counted and converted to Log10 (CFU/mL) for analysis.

### 2.4. MB fluorescence analysis

In order to verify the influence of glucose on MB uptake, the amount of MB that remained in the solution after cell incubation was measured. Inocula of *C. albicans* strains were incubated with 100 µM MB and 50 mM glucose or with only 100 µM MB, as previously described. Then inocula were centrifuged at 4000 *g* for 3 min and 100 µL of supernatant from each group was placed in wells of 96-well microtiter plates for analysis. MB fluorescence was measured in a spectrophotometer (SpectraMax® Plus 384, Molecular Devices, CA, USA) with excitation at 532 nm and emission at 690 nm [17]. For each strain, we evaluated the supernatant of inocula incubated with MB combined or not with glucose. Eight samples from each group were analyzed.

### 2.5. Statistics

The CFU counts presented normal distribution (Shapiro-Wilk test), and they were statistically analyzed using One-way analysis of variance (ANOVA). Mean comparison was performed with Tukey's test and the significance level was set at 5%.

## 3. Results

Cells incubated with glucose and MB but without irradiation did not present any reduction in the number of viable cells when compared to the control groups ($p > 0.05$). In addition, PACT groups with or without glucose did not show a significant killing effect after 1 min of irradiation in all strains ($p > 0.05$, Fig. 1).

Increasing the time of irradiation, we observed distinct behavior when comparing different strains. After 3 min of irradiation, ATCC 10,231 showed complete inactivation of cells whether incubated or not with glucose. On the other hand, the YEM strains presented moderate to high killing effect, depending on the strain and glucose incubation ($p < 0.05$).

### 3.1. Glucose effect

The presence of 50 mM glucose did not affect the PACT killing effect in this model. However, the YEM 12 strain was protected from PACT. Glucose affected PACT only in YEM12. After 3 min of irradiation, YEM12 cells incubated with glucose showed a 1-log decrease in viable cells ($p < 0.05$), and after 6 min, an additional 4-log decrease ($p < 0.05$). For YEM13, glucose did not promote significant difference in the killing effect ($p > 0.05$). Furthermore, glucose slight affected PACT only in YEM 15 and increased 1 log of the number of viable cells ($p < 0.05$).

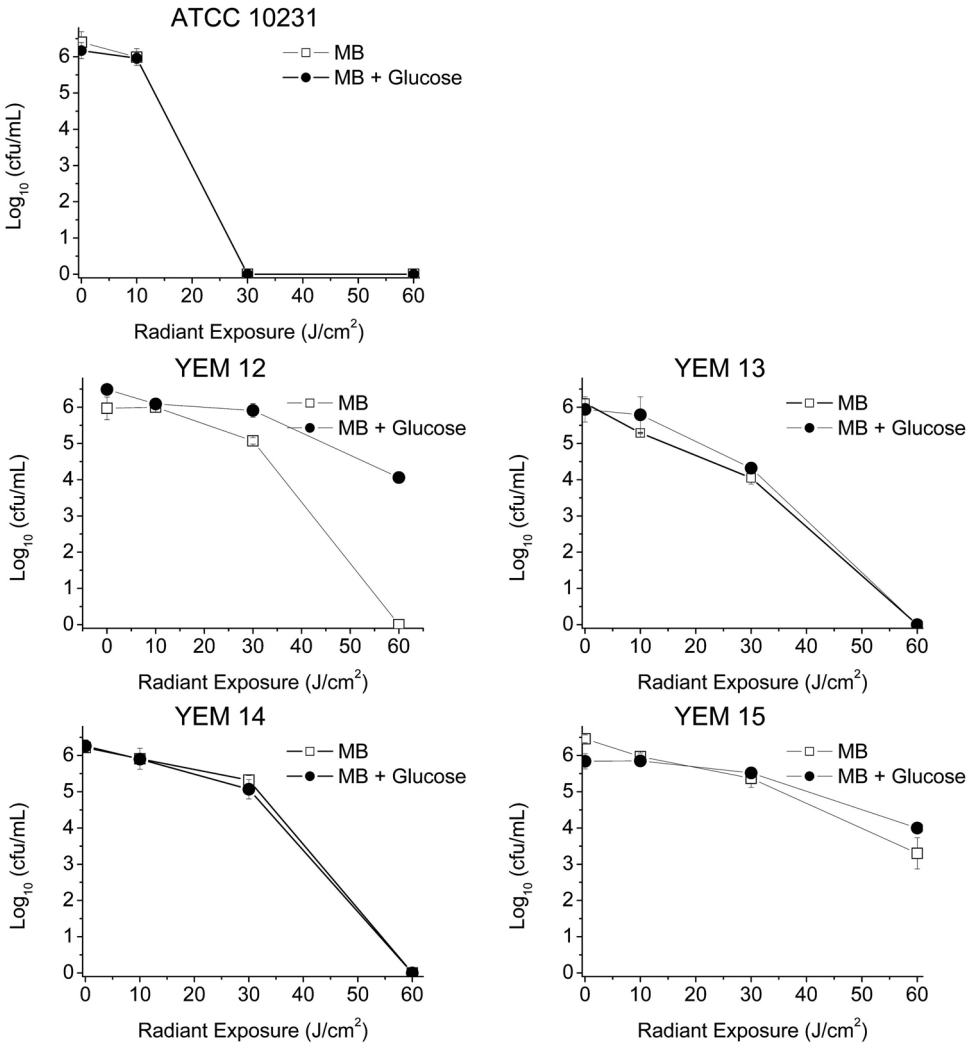


**Fig. 1.** Killing effect of PACT on different strains of *C. albicans*. Data are mean and standard deviation of $\log_{10}$ (cfu/mL) of yeast cell survivors following PACT. Each section of data is the mean of at least 3 experiments repeated on different days. YEM13 is a strain Overexpressing *MDR1* created form YEM12 wild type. And YEM15 is a strain Overexpressing *CDR1*, *CDR2*, derived from YEM14 wild type.

### 3.2. Efflux systems

Regarding the presence of MFS transporter, comparison between YEM12 and YEM13 showed that overexpression of MFS system (YEM13) improved the effect of PACT (Fig.1). After 3 min of irradiation YEM13 showed an enhancement of cell killing of about 1 log compared to YEM12 ($p < 0.05$). For ABC systems, overexpression of ABC efflux pumps (YEM15) protected the yeast from killing effect (Fig.1). YEM14 cells were totally inactivated (a 6-log reduction) after 6 min of irradiation, whereas YEM15 showed about a 2 log of cell reduction ($p < 0.05$). In regards to antimicrobial inactivation of MB-mediated PACT, overexpression of *MDR1* favors cell death while the overexpression of *CDR1* and *CDR2* inhibited PACT killing. The presence of glucose conferred a protective effect mainly on the expressing strains of the MFS system transporters.

The fluorescence of MB in suspension was evaluated to measure the amount of MB uptake by the cells. Fluorescence signal was detected in all samples (Fig. 2) and as a higher level of fluorescence signal appears, more MB was found outside the cell.

### 3.3. Glucose effect

Comparing samples incubated or not with glucose from the same strain, we observed that glucose increased the amount of MB outside the yeast cells in ATCC 10,231, YEM12, and YEM15 ($p < 0.01$).

### 3.4. Efflux systems

Comparison between YEM12 and YEM13 showed that overexpression of *MDR1* did not affect MB uptake in the presence or absence of glucose. However, an increase in MB outside the cells was observed when comparing YEM14 and YEM15 with glucose ($p < 0.01$).

## 4. Discussion

There are a great number of published studies exploring different PSs, irradiation parameters and new strategies to enhance PACT effects in pathogenic microorganism. Besides, there is little information concerning the influence of membrane transport systems and cells substrates in PACT. Glucose have been shown to alter MB–mediated PACT on *Streptococcus mutans* suspension and biofilm [18] and in *C. albicans* biofilm [19]. In the present study we confirmed that glucose can affect MB uptake and PACT killing in *C. albicans* and we demonstrate the role of ABC (Cdr1p and Cdr2p) and MFS (Mdr1p) transport proteins on these effects.

Regarding the impact of ABC and MFS systems in the antimicrobial activity of MB-mediated PACT, the comparison between YEM14 and YEM15 showed that overexpression of *CDR1* and *CDR2* inhibited the

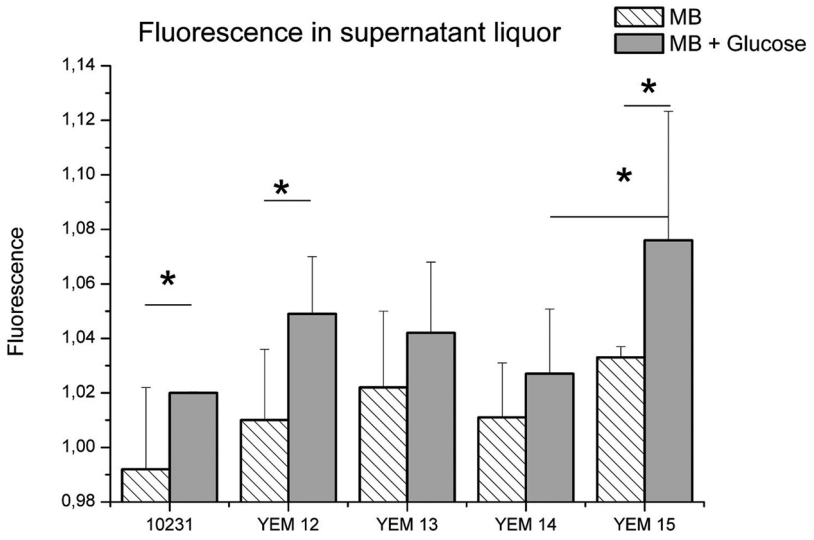


**Fig. 2.** Fluorescence data in supernatant liquor. Data are mean and standard deviation. (*) symbol means statistically significant difference between indicated groups ($p < 0.05$). Less fluorescence indicates that there is more photosensitizer inside the yeast.

antimicrobial activity of MB-mediated PACT. In contrast, overexpression of *MDR1* (YEM13) slightly favored cell death after 3 min of irradiation. These results can be explained by the fact that ABC pumps are responsible for MB efflux, whereas MFS systems are implicated in both the influx and efflux of this PS [5].

Furthermore, glucose promotes different behavior according to the characteristics of the yeast. In a strain highly susceptible to PACT as ATCC 10,231, glucose reduced MB uptake without affecting PACT efficiency. On the other hand, in YEM15 glucose caused a reduction of MB uptake and also inhibited the killing effect. In addition, glucose promoted no effect on YEM14. The nutrient glucose is an important carbon sources used by *C. albicans* to provide energy [20]. Therefore, the metabolism of glucose can increase energy availability for MB efflux via ABC system and this effect was only observed when *CDR1* and *CDR2* were overexpressed (YEM15).

Glucose promoted a pronounced effect on YEM12, but not in its overexpressing *MDR1* parent strain, YEM13. Following incubation with glucose, YEM12 presented less MB uptake and the number of cells killed by PACT reduced by 4 logs. The reduction of MB uptake could be a result of decreased influx, increased efflux or less monomer in the solution to provide fluorescence signal. Suzuki et al. showed that glucose did not alter optical characteristics of MB in solution [19], and monomer/dimer relation did not change. Since Mdr1p can transport MB in and out of the cell cytoplasm [5] and the transport is independent of ATP energy, a plausible explanation is that glucose might inhibit MB entrance. Regarding the reduced antimicrobial effect, two mechanisms could explain this result; the glucose increased the antioxidant enzymes generation by enhancing mitochondrial respiration and ROS formation [21], and glucose enhanced oxidative stress resistance by up regulating of *CAP1* and *HOG1* genes. *CAP1* encodes a transcriptional activator required for resistance to oxidative stress and Hog1 encodes the protein kinase that regulates stress responses [22]. In YEM13 glucose did not alter MB uptake, then we can hypothesize that the higher number of MFS transporters allowed a continuously influx of MB during irradiation and the ROS generated by PACT suppressed the antioxidant effects. Nevertheless, further studies are necessary to verify these hypotheses.

One important aspect of a commensal microorganism, i.e. *C. albicans,* is to adapt to environmental characteristics in order to survive to different conditions of host niches. This ability requires metabolic and structural changes [23] that might affect PACT. It was demonstrated that *C. albicans* presents different susceptibility to PACT according to the phase of cell growth [24]. This finding suggests that changes in metabolism, besides in cell structure, may affect the photodynamic efficiency. Furthermore, *C. albicans* can assimilate sugars and alternative carbon sources simultaneously and this ability allows this yeast to colonize and infect niches with limiting or high sugar concentrations [23]. Under this scenario, our results indicate that glucose availability may alter MB uptake and PACT killing and this effect is related to strain characteristics. Due to metabolic flexibility of *C. albicans*, the sugar availability of the infected area needs to be considered for further consideration of mechanistic ways for photodynamic antimicrobial chemotherapy.

The understanding of how yeasts incorporate and/or exclude photosensitizing agents and the influence of nutrients is important for further consideration of mechanistic ways for photodynamic antimicrobial chemotherapy.

## 5. Conclusions

The overexpression of ABC efflux system protected yeast cell against photodynamic inactivation, whereas no effect was observed due to overexpression of MFS system. Glucose can influence the incorporation of MB in *C. albicans* and it promoted partial effect on photodynamic efficiency according to the characteristics of the strain. Thus, the type of metabolism can be a determinant of the photodynamic effect in *C. albicans*.

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