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Optimization of cellular uptake of zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)] phthalocyanine for maximal photoinactivation of *Candida albicans*

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ABSTRACT

Cellular uptake and photodynamic action of zinc(II) 2,9,16,23-tetrakis[4-(N-methylpyridyloxy)]phthalocyanine ($ZnPPc^{4+}$) was examined in *Candida albicans*. *In vitro* investigations showed that $ZnPPc^{4+}$ was rapidly bound to *C. albicans* cells. The binding of phthalocyanine to cells was dependent on $ZnPPc^{4+}$ concentrations (1–10 μM) and cells densities (10^6–10^8 cells mL^{-1}). A high amount of $ZnPPc^{4+}$ retained in the cells after two washing steps, indicating a strong interaction between the photosensitizer and *C. albicans*. The uptake was temperature dependent, although the difference between 37 °C and 4 °C was about 10 %. Also, the amount of $ZnPPc^{4+}$ bound to *C. albicans* was affected when the cells were incubated for a longer time with azide and 2,4-dinitrophenol (DNP) prior to treatment with $ZnPPc^{4+}$. Cell survival after irradiation was dependent on the irradiation period, $ZnPPc^{4+}$ concentration and cells density. Photoinactivation of *C. albicans* cells was elevated even after two washing steps. The strong dependence of uptake on cell density reveals the strength and avidity of the binding of $ZnPPc^{4+}$ to *C. albicans* cells. The accumulation behaviour of $ZnPPc^{4+}$ suggests that mainly an affinity-mediated binding mechanism can be involved. Therefore, $ZnPPc^{4+}$ is an interesting phthalocyanine for photodynamic inactivation (PDI) of yeasts in liquid suspensions.

Introduction

The incidence of superficial and deep-seated fungal infections has increased markedly over the last 20 y (Lai *et al.* 2008). Several reasons have been proposed, which include the increasing use of antineoplastic and immunosuppressive drugs, broad-spectrum antibiotics, prosthetic devices and grafts, and more aggressive surgery. Patients with burns, neutropenia, HIV infection, and pancreatitis are also predisposed to fungal infection. *Candida albicans* is commensal and a constituent of the normal gut flora comprising microorganisms that live in the human mouth and gastrointestinal tract. However, *Candida* can cause a wide variety of infections on mucosal surfaces under certain conditions (Tsai *et al.* 2013). The majority

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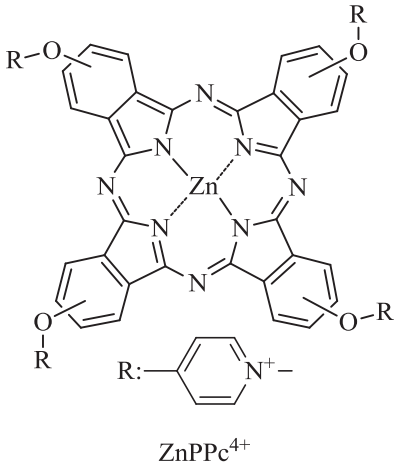
of *C. albicans* isolates causing disease in AIDS patients are derived from strains originally associated with commensal infections. Recent development of new antifungal agents has significantly contributed to the successful treatment. However, the pharmacokinetics of antifungal agents can be altered in a number of disease states, including critical illness. Moreover, inadequate dosing may contribute to treatment failure and the emergence of resistance (Sinnollareddya *et al.* 2012). In this way, photodynamic inactivation (PDI) of microorganisms represents an alternative approach to treat fungal infections (Luksiene & Zukauskas 2009; Pereira Gonzales & Maisch 2012). The basic principle of PDI is the administration of a photosensitizer, which is preferentially accumulated in the microbes. Subsequent irradiation with visible light under aerobic conditions results in the formation of reactive oxygen species (ROS) through either electron transfer (type I) or energy transfer (type II) reactions. These ROS can react with many cellular components that induce oxidative processes leading to cell death (Pereira Gonzales & Maisch 2012).

The fungal cell wall provides structure to the cell and protects it from the environment. It is composed mainly of β-glucans, chitin, and mannoproteins, which account for the rigidity of the wall and for the fungal morphology (Osumi 1998; Marcilla *et al.* 1998). Thus, a relatively thick layer of β-glucan and chitin leads to a permeability barrier, resulting in some resistance to the photoinactivation. Therefore, the efficiency of the photosensitized process is markedly more pronounced when used agents to enhance their penetration into the inner cell area. Photodynamic activity of zinc(II) tetrakis-(3-methylpyridyloxy)phthalocyanine (ZnPcMe) and zinc(II) tetrakis-(4-sulfophenoxy)phthalocyanine (ZnPcS) were previously compared in different microorganisms. The yeast *C. albicans* was effectively photodynamically inactivated with the cationic ZnPcMe. Also, a water-soluble cationic phthalocyanine complex of silicon showed a potential value for PDI of *C. albicans* in suspension (Mantareva *et al.* 2011).

In the present investigation, we evaluated the cellular uptake and photodynamic activity of zinc(II) 2,9,16,23-tetrakis [4-(*N*-methylpyridyloxy)]phthalocyanine ($ZnPPc^{4+}$) (Scheme 1) in *C. albicans* cells. Studies in different biomimetic media and in bacterial cells have shown that $ZnPPc^{4+}$ is an effective photosensitizer (Spesia *et al.* 2009; Spesia *et al.* 2010). However, the uptake of photosensitizers by fungal cells is an important feature for the efficacy of photoinactivation (Cormick *et al.* 2009). Therefore, the amount of $ZnPPc^{4+}$ bound to *C. albicans* cells was examined under different conditions, such as photosensitizer concentrations, incubation times, washing steps, cellular densities, and temperatures. Also, the uptake was evaluated in presence metabolic inhibitors (Boiron *et al.* 1987; Bliss *et al.* 2004). Taking into account these conditions of uptake, the photodynamic action of $ZnPPc^{4+}$ was examined to inactivate *C. albicans* cells.



$ZnPPc^{4+}$

Scheme 1 – Molecular structures of $ZnPPc^{4+}$.

Materials and methods

General

Absorption and fluorescence spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan) and on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon, Edison, NJ, USA), respectively. Quartz cuvettes of 1 cm path length were used at room temperature. Absorptions of cell cultures were determined at 550 nm in a Barnstead Turner SP-830 spectrophotometer (Dubuque, IA, USA). Irradiation was performed using a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. A wavelength range between 350 and 800 nm was selected by optical filters. The light fluence rate at the treatment site was 30 mW cm^{-2} (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA). Chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Solvents (GR grade) from Merck (Darmstadt, Germany) were distilled. Ultrapure water was obtained from Labconco (Kansas, MO, USA) equipment model 90901-01.

Phthalocyanine

$ZnPPc^{4+}$ was synthesized as previously described (Scalise & Durantini 2005). A stock solution 0.5 mM $ZnPPc^{4+}$ was prepared by dissolution in 1 mL of *N*,*N*-dimethylformamide (DMF). The concentration was established by spectroscopy, considering the value of molar extinction coefficient, $\varepsilon = 1.1 \times 10^5$ M^{-1} cm^{-1} at 678 nm in DMF (Scalise & Durantini 2005).

Yeast strain and preparation of cultures

The strain of *Candida albicans* PC31 was recovered from human skin lesion and it was previously characterized and identified (Cormick *et al.* 2009). Fungal cells were grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (3 mL) at 37 °C to stationary phase. A portion (1 mL) of this culture was dissolved in 3 mL Sabouraud broth. Then, cells were harvested by centrifugation of broth cultures (3000 rpm for 15 min) and re-suspended in 4 mL of 10 mM phosphate-buffered saline (PBS, pH = 7.0). This procedure was used to obtain ~10^7 colony forming units (CFU) mL^{-1}. After that, cells were appropriately diluted to obtain ~10^6 CFU mL^{-1} or

concentrated to get ~10^8 CFU mL^{-1} in PBS. In all experiments, 2 mL of cell suspensions were used in Pyrex brand culture tubes (13 × 100 mm). After treatments, cells were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable *C. albicans* cells were observed on Sabouraud agar plates after ~48 h incubation at 37 °C and quantified as the number of CFU.

Binding of phthalocyanine to Candida albicans *cells*

Candida albicans cells (2 mL, ~10^6–10^8 CFU mL^{-1}) in PBS were incubated in dark at 37 °C with 5 and 10 μM of $ZnPPc^{4+}$ for different times. Cell suspensions were centrifuged (3000 rpm for 15 min) and the cell pellets were re-suspended in 2 mL of 2 % aqueous SDS (sodium dodecyl sulfate), incubated overnight at 4 °C and sonicated for 30 min. The concentration of $ZnPPc^{4+}$ in the SDS aqueous medium was determined by spectrofluorimetry (λ_{exc} = 670 nm, λ_{em} = 684 nm). The fluorescence intensities obtained from each sample were referred to the total number of cells contained in the cell suspension. The concentration of $ZnPPc^{4+}$ in this sample was estimated by comparison with a calibration curve using a standard solutions of the $ZnPPc^{4+}$ in 2 % SDS ([$ZnPPc^{4+}$] ~0.005–0.5 μM). Binding experiments at 4 °C were performed as described above using a thermostated bath. Cell suspensions were previously treated at this temperature for 30 min. Sodium azide (10 mM) and 2,4-dinitrophenol (DNP, 10 mM) were added to yeast suspensions from 2.0 M stock solutions in water and 1.0 M stock solutions in DMF, respectively. This amount of sodium azide was not toxic to *C. albicans* cells. The cells were incubated with 5 μM $ZnPPc^{4+}$ for 30 min at 37 °C in dark and then 10 mM sodium azide and 10 mM DNP were added and the cells were further incubated for 30 min. Also, the cells were incubated with 10 mM azide and 10 mM DNP for different times (30, 60, and 120 min) before treatment with 5 μM $ZnPPc^{4+}$ for 30 min in dark at 37 °C.

Photoinactivation of Candida albicans *cells in PBS suspension*

Candida albicans cells (2 mL, ~10^6–10^8 CFU mL^{-1}) in PBS were incubated with a determined concentration of $ZnPPc^{4+}$ (1, 2.5, 5, and 10 μM) for different times (2.5, 15, and 30 min) in the dark at 37 °C. After that, the cultures were exposed to visible light for different periods of irradiation (15 and 30 min). Cells were serially diluted with PBS and the number of colonies formed was counted as described above.

Controls and statistical analysis

Control experiments were performed in presence of $ZnPPc^{4+}$ in the dark and in the absence of $ZnPPc^{4+}$ with cells irradiated and in the dark. The amount of DMF used in each experiment was not toxic to *Candida albicans* cells. Three values were obtained per each condition and each experiment was repeated separately three times. The unpaired *t*-test was used to establish the significance of differences between groups. Differences were considered statistically significant with a confidence level of 95 % ($p < 0.05$). Data were represented as the mean ± standard deviation of each group.

Results

Binding of $ZnPPc^{4+}$ *to* Candida albicans *cells*

The ability of cationic $ZnPPc^{4+}$ to bind to *Candida albicans* was determined in cell suspensions of ~10^6 cells mL^{-1} in PBS. For this purpose, *C. albicans* cells were incubated with 5 and 10 μM $ZnPPc^{4+}$ for different times (15, 30, and 60 min) at 37 °C in the dark. The amount of $ZnPPc^{4+}$ recovered after each incubation period is shown Fig 1. As can be observed, the phthalocyanine reached the highest value of cell-bound sensitizer at short time (<15 min). An increase in the amount of photosensitizer bound to *C. albicans* cells was not observed prolonging the incubation time to 60 min. Under this condition, the binding of $ZnPPc^{4+}$ reached saturation values of 1.8 and 4.6 nmol 10^{-6} cells for *C. albicans* incubated with 5 and 10 μM, respectively.

Moreover, the amount of cell-bound $ZnPPc^{4+}$ was evaluated after one and two washed steps (Fig 1). The quantity of phthalocyanine, expressed as nmol concentration per 10^6 cells, decreased slightly when the cells are washed with PBS. After 30 min incubation and one washing steps, values of 1.6 and 4.1 nmol 10^{-6} cells were found using 5 and 10 μM $ZnPPc^{4+}$, respectively. After a second washing step, these amounts of $ZnPPc^{4+}$ diminished to 1.4 and 3.8 nmol 10^{-6} cells when cells were washed two times with PBS.

On the other hand, the binding of $ZnPPc^{4+}$ to *C. albicans* was analysed for different cell densities (10^6–10^8 cells mL^{-1}) incubated with 5 and 10 μM phthalocyanine for 15 and 30 min at 37 °C in the dark. The results for 10^7 and 10^8 cells mL^{-1} are shown in Fig 2A. The uptakes of $ZnPPc^{4+}$ by cells treated

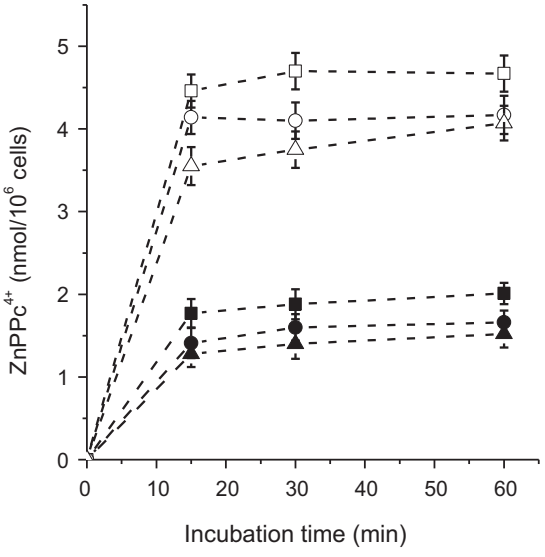


Fig 1 – Amount of $ZnPPc^{4+}$ recovered from *C. albicans* cell suspensions in PBS (~10^6 CFU mL^{-1}) treated with 5 μM phthalocyanine after 0 (■), 1 (●), and 2 (▲) washing steps and with 10 μM phthalocyanine after 0 (□), 1 (○), and 2 (Δ) washing steps at 37 °C in dark for different incubation times. Values represent mean ± standard deviation of three separate experiments.

with 10 μM phthalocyanine for 30 min were 0.28 and 0.049 nmol 10^{-6} cells for cultures of 10^7 and 10^8 cells mL^{-1}, respectively. These values decreased to 0.12 for 10^7 cells mL^{-1} and 0.031 nmol 10^{-6} cells for 10^8 cells mL^{-1}. The results of phthalocyanine uptake by *C. albicans* cells using different cell densities are summarized in Fig 2B. At both $ZnPPc^{4+}$ concentrations, the phthalocyanine bound to the fungal cells decreased with increasing of cell density.

The effect of $ZnPPc^{4+}$ concentration on the amount of photosensitizer bound to *C. albicans* cells was compared in Fig 3 for cultures of 10^6 cells mL^{-1}. The tendency was quite linear over the range of concentrations used (1–10 μM).

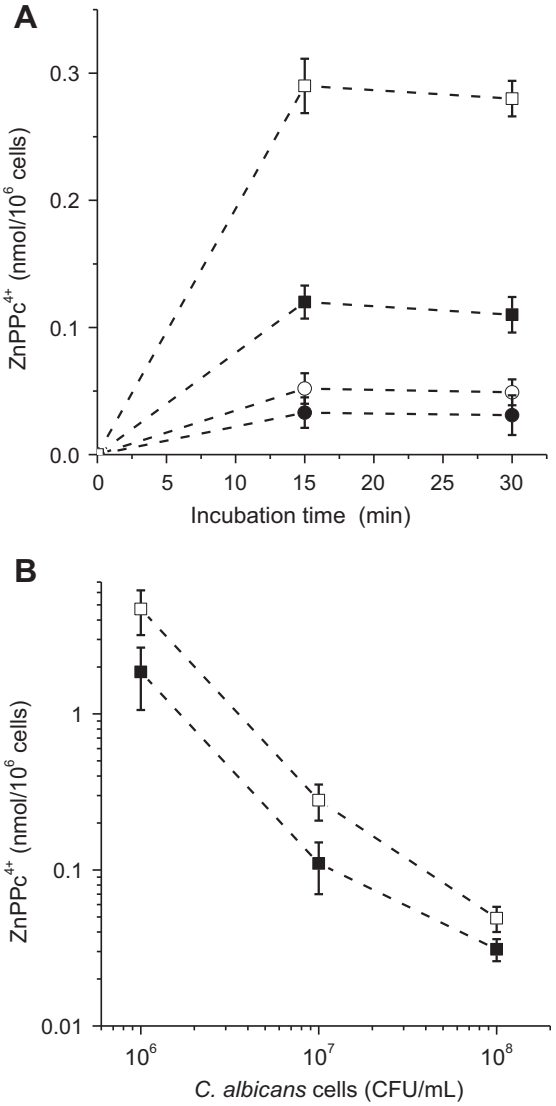


Fig 2 – (A) Amount of $ZnPPc^{4+}$ recovered from *C. albicans* $\sim 10^7$ CFU mL^{-1} cell suspensions in PBS treated with 5 μM (■) and 10 μM (□) phthalocyanine and $\sim 10^8$ CFU mL^{-1} cell suspensions incubated with 5 μM (●) and 10 μM (○) phthalocyanine at 37 °C in dark for different incubation times; (B) amount of $ZnPPc^{4+}$ bound to cells with different cell densities and incubated for 30 min with 5 μM (■) and 10 μM (□). Values represent mean ± standard deviation of three separate experiments.

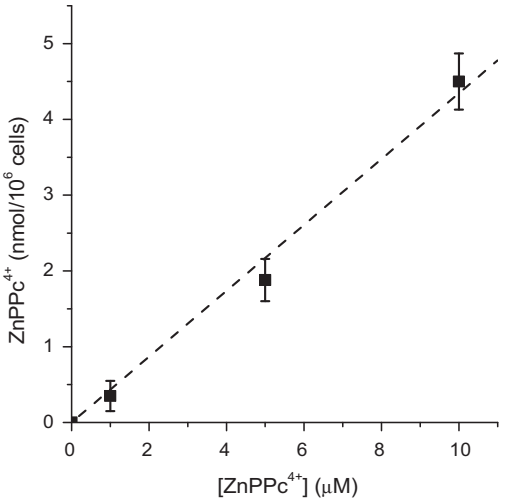


Fig 3 – Amount of $ZnPPc^{4+}$ recovered from *C. albicans* cell suspensions in PBS ($\sim 10^6$ CFU mL^{-1}) treated with different phthalocyanine concentration for 30 min in dark at 37 °C. Values represent mean ± standard deviation of three separate experiments.

Also, the effect of temperature at 37 and 4 °C was analysed on the uptake of $ZnPPc^{4+}$ by the *C. albicans* cells. As can be observed in Fig 4, the uptake of $ZnPPc^{4+}$ was similar at both temperatures. Although, the binding of phthalocyanine to the cells was decreased at 4 °C with respect to 37 °C.

The effect of sodium azide and DNP addition on the uptake of $ZnPPc^{4+}$ was studied under different conditions. As shown in Fig 5, the amount of $ZnPPc^{4+}$ recovered from *C. albicans* cell

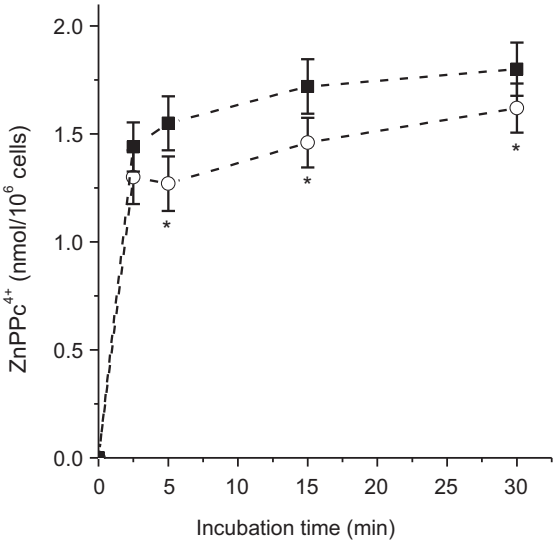


Fig 4 – Amount of $ZnPPc^{4+}$ recovered from *C. albicans* cell suspensions in PBS ($\sim 10^6$ CFU mL^{-1}) treated with 5 μM phthalocyanine at 37 °C (■) and 4 °C (○) in dark for different incubation times. Values represent mean ± standard deviation of three separate experiments ($^*p < 0.05$, compared with 37 °C).

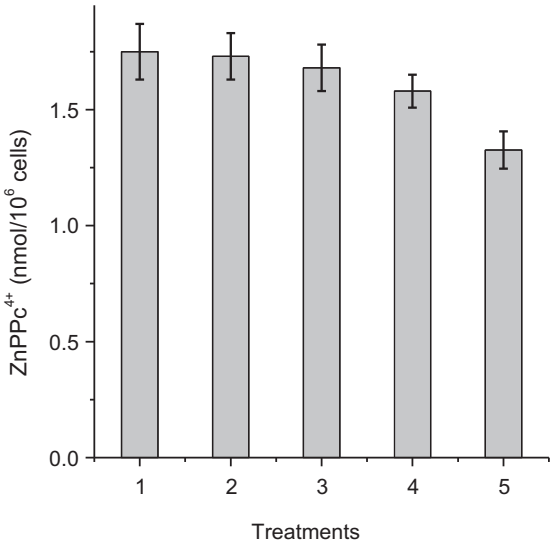


Fig 5 – Amount of $ZnPPc^{4+}$ recovered from *C. albicans* cell suspensions in PBS ($\sim 10^6$ CFU mL^{-1}) incubated with 1) 5 μM phthalocyanine for 30 min in dark at 37 °C; 2) 5 μM phthalocyanine for 30 min and then 10 mM sodium azide and 10 mM DNP for 30 min in dark at 37 °C; 3) 10 mM sodium azide and 10 mM DNP for 30 min and then with 5 μM phthalocyanine for 30 min in dark at 37 °C; 4) idem 3 but incubating for 60 min with azide and DNP; 5) idem 3 but incubating for 120 min with azide and DNP. Values represent mean ± standard deviation of three separate experiments.

suspensions in PBS ($\sim 10^6$ cells mL^{-1}) incubated with 5 μM phthalocyanine for 30 min followed by incubation with 10 mM sodium azide and 10 mM DNP for 30 min in dark at 37 °C was close similar to that using only 5 μM phthalocyanine. Also, cells were treated with 10 mM of both azide ion and DNP for different times (30, 60, and 120 min) before treatment with 5 μM $ZnPPc^{4+}$ for 30 min in dark at 37 °C. Under these conditions, the amount of $ZnPPc^{4+}$ mainly diminished to 1.6 and 1.3 nmol 10^{-6} cells with a preincubation with azide and DNP of 60 and 120 min, respectively, before the addition of phthalocyanine (Fig 5, lines 4 and 5).

Photosensitized inactivation of Candida albicans *cell suspensions in PBS*

Photoinactivation of *Candida albicans* was first evaluated in PBS cell suspensions ($\sim 10^6$ cells mL^{-1}) treated with different concentrations (1–10 μM) of $ZnPPc^{4+}$ for 30 min in dark at 37 °C. The results are shown in Fig 6. No toxicity was detected for the cells in PBS incubated with 1–10 μM phthalocyanine for 30 min in dark. Moreover, control experiments showed that the viability of *C. albicans* was not modified by illumination alone. Therefore, the cell inactivation obtained after irradiation of the *C. albicans* treated with $ZnPPc^{4+}$ was due to the photosensitization effect of the phthalocyanine. A rapid decrease of *C. albicans* survival was observed after irradiation with visible light. An irradiation time of 15 min was sufficient to produce a 5 log decrease in the cell viability treated with 10 μM $ZnPPc^{4+}$. At this irradiation time, it was possible to observe a clear dependence between the photoinactivation and the concentration of phthalocyanine (1–10 μM). After 30 min irradiation, the viability of *C. albicans* cells was effective even using 1 μM $ZnPPc^{4+}$ to photoinactivate 4 log of *C. albicans* cells. An increase in the phthalocyanine concentration between 5 and 10 μM is not accompanied by an enhancement in the PDI efficiency, causing >5 log decreases.

On the other hand, PDI of *C. albicans* treated with 5 μM $ZnPPc^{4+}$ was evaluated after different washing steps with PBS. As can be observed in Fig 7, photoinactivations were very similar for cells washed once or two times. The phototoxic action after washing was lower for ~1 log than that found without washing step. These results are in agreement with a small decrease in the amount of $ZnPPc^{4+}$ bound to the cells after washing (Fig 1).

The effect of different incubation periods with the photosensitizer was investigated on the survival of *C. albicans*. Cells were incubated with 5 μM $ZnPPc^{4+}$ in dark at 37 °C and irradiated for 30 min. As shown in Fig 8, a slightly lower inactivation was found for cells incubated for 2.5 min, while similar values were obtained for cells treated for 15 and 30 min.

Moreover, photoinactivation of *C. albicans* induced by $ZnPPc^{4+}$ was analysed using different cellular densities. The results for 10^6–10^8 cells mL^{-1} treated with 5 μM phthalocyanine are shown in Fig 9. The effectiveness of PDI decreases significantly with an increase in the cell density. Thus, cell suspension of 10^7 cells mL^{-1} incubated with $ZnPPc^{4+}$ required over 30 min of irradiation to photoinactivate 2.5 log of *C. albicans*. In particular, when 10^8 cells mL^{-1} was incubated with 5 μM $ZnPPc^{4+}$ and irradiated for 30 min, the cell survival of *C. albicans* was practically not changed with respect to the untreated control. Also, 10^7 cells mL^{-1} were treated with 50 μM $ZnPPc^{4+}$ for 30 min in dark at 37 °C and then irradiated for 30 min with visible light. This procedure produced a 3 log decrease in the cell viability.

Discussion

The structure of $ZnPPc^{4+}$ contains four cationic *N*-methylpyridyloxy groups attached to the phthalocyanine macrocycle by an ether bond (Scheme 1). This spacer provides mobility to the charges, facilitating the interaction with the *Candida albicans* cell envelop. Also, this charges distribution has minimal influence on the electronic properties of the phthalocyanine. The absorption spectrum of $ZnPPc^{4+}$ is characterized by the typical *Soret* (374 nm) and Q-band (678 nm) with a high absorption coefficient in the visible region (Scalise & Durantini 2005). $ZnPPc^{4+}$ emits two bands at 687 and 756 nm with a fluorescence quantum yield of 0.22 in DMF. This property allows its detection and quantification in biological media even at very low concentrations (Spesia *et al.* 2009). Moreover, $ZnPPc^{4+}$ presents a high photodynamic activity with singlet molecular oxygen quantum yield of 0.59 in DMF (Scalise & Durantini 2005).

In vitro experiments with *C. albicans* (10^6 cells mL^{-1}), it was found that the amount of cell-bound $ZnPPc^{4+}$ was not dependent on the incubation time between 15 and 60 min at 37 °C

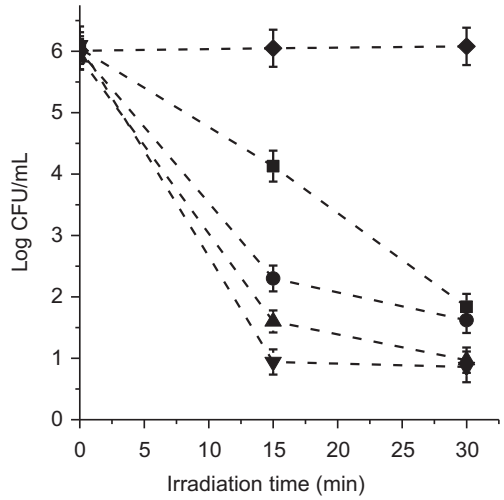


Fig 6 – Survival curve of *C. albicans* (~10^6 CFU mL^{-1}) incubated with 1 (■), 2.5 (●), 5 (▲) and 10 (▼) μM $ZnPPc^{4+}$ for 30 min at 37 °C in dark and exposed to visible light for different irradiation times. Control culture untreated (◆). Values represent mean ± standard deviation of three separate experiments.

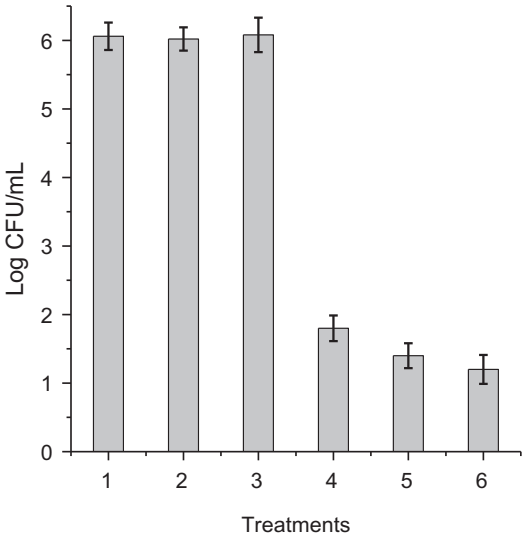


Fig 8 – Survival of *C. albicans* (~10^6 CFU mL^{-1}) incubated with 5 μM $ZnPPc^{4+}$ in dark at 37 °C and exposed to visible light for 30 min; 1) control cells in dark; 2) control cells irradiated; 3) control cells treated with $ZnPPc^{4+}$ and keeping in dark; 4) cells incubated with $ZnPPc^{4+}$ for 2.5 min and irradiated; 5) cells incubated with $ZnPPc^{4+}$ for 15 min and irradiated; 6) cells incubated with $ZnPPc^{4+}$ for 30 min and irradiated. Values represent mean ± standard deviation of three separate experiments.

(Fig 1). A similar behaviour of $ZnPPc^{4+}$ bound to cells was previously found for *Escherichia coli* and *Streptococcus mitis* treated with $ZnPPc^{4+}$ (Spesia *et al.* 2010). Moreover, the uptake of $ZnPPc^{4+}$ by *C. albicans* can be compared with those obtained for 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammonium phenyl)porphyrin ($TMAP^{4+}$) (Cormick *et al.* 2009). Under similar conditions, this tetracationic porphyrin reached a value of 1.35 nmol 10^{-6} cells, when the cell suspensions were incubated with 5 μM $TMAP^{4+}$ for 30 min. In the same way, 1.70 nmol 10^{-6} cells was found using 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin ($TMPyP^{4+}$) (Quiroga *et al.* 2010). Thus, the binding of $ZnPPc^{4+}$ to *C. albicans* was quite similar to those of tetracationic porphyrins.

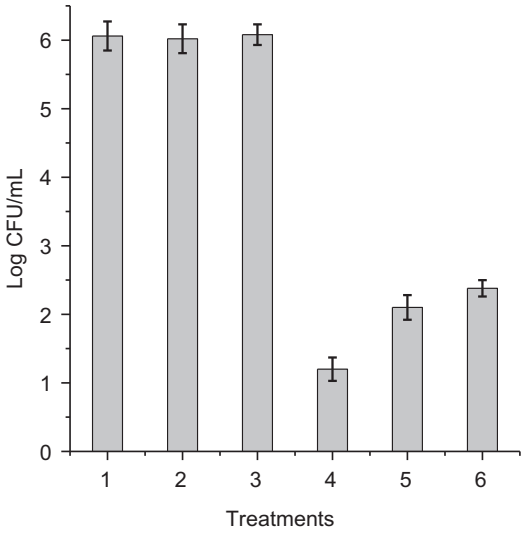


Fig 7 – Survival of *C. albicans* (~10^6 CFU mL^{-1}) incubated with 5 μM $ZnPPc^{4+}$ in dark for 30 min at 37 °C and exposed to visible light for 30 min; 1) control cells in dark; 2) control cells irradiated; 3) control cells treated with $ZnPPc^{4+}$ and keeping in dark; 4) cells treated with $ZnPPc^{4+}$ and irradiated; 5) cells treated with $ZnPPc^{4+}$, washed once and irradiated; 6) cells treated with $ZnPPc^{4+}$, washed two times and irradiated. Values represent mean ± standard deviation of three separate experiments.

After two washing steps, ~80 % of the initial $ZnPPc^{4+}$ still remains bound to the cells, for both *C. albicans* incubated with 5 or 10 μM phthalocyanine (Fig 1). In contrast, it was observed that $TMPyP^{4+}$ decreased to 30 % of initial value after two washes (Quiroga *et al.* 2010). Therefore, a significant amount of $ZnPPc^{4+}$ still remains in the cells after two washing steps indicating a strong interaction between the photosensitizer and cells.

The amount of cell-bound $ZnPPc^{4+}$ was dependent on the *C. albicans* cell density, decreasing as the density increases (Fig 2). A similar behaviour was found for the binding of $TMAP^{4+}$, reaching values of 0.11 for and 0.01 nmol 10^{-6} cells for cultures of 10^7 and 10^8 cells mL^{-1} (Cormick *et al.* 2009). Also, the drug uptake dependency with an inverse behaviour to the cell density was observed using a tetracationic phthalocyanine, ZnPcMe (Mantareva *et al.* 2007). Similarly, the uptake of a poly-L-lysine chlorin(e6) conjugate (pL-ce6) showed a marked inverse dependence on cell density (Demidova & Hamblin 2005). Decreasing the *C. albicans* cell density 10-fold gave a corresponding 10-fold increase in cell uptake of pL-ce6. For toluidine blue O (TBO), there was again an inverse dependence of uptake on cell density but not as marked as that

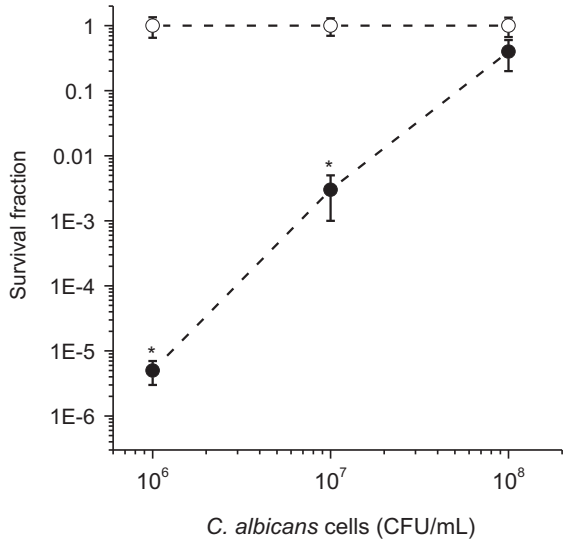


Fig 9 – Survival curve of *C. albicans* with different cell densities incubated with 5 μM $ZnPPc^{4+}$ for 30 min at 37 °C in dark and exposed to visible light for 30 min (30 mW cm^{-2}). Control culture untreated (○). Values represent mean ± standard deviation of three separate experiments (*$p < 0.05$, compared with untreated cells).

seen with pL-ce6. With increasing cell density, $ZnPPc^{4+}$ molecules are distributed over a greater number of cells and therefore, the amount of phthalocyanine bound to the fungi decreased with increasing of cell density.

The most microbial species demonstrate two mechanisms of drug uptake: a diffusion-controlled process and an affinity-mediated binding (Demidova & Hamblin 2005). The degree of uptake of $ZnPPc^{4+}$ by *C. albicans* was dependent on incubation concentration of the phthalocyanine. The effect of $ZnPPc^{4+}$ concentration (Fig 3) on the uptake of photosensitizer by *C. albicans* using cellular densities of 10^6 cells mL^{-1} showed a tendency quite linear over the range of concentrations used (1–10 μM). Therefore, apparently non-saturation in the intracellular photosensitizer concentration occurs, at least over this concentration range. Moreover, the effect of temperature on the binding of photosensitized to cells was investigated at both 4 and 37 °C. The results showed that the uptake of $ZnPPc^{4+}$ by the *C. albicans* cells was significantly dependent on temperature (Fig 4). Even so, this difference in the uptake of photosensitizer at both temperatures is about 10 %. These results suggest that the uptake could involve an important contribution of a diffusional process. On the other hand, metabolic inhibitors were added to *C. albicans* cells to evaluate the active transport system. Sodium azide was used an inhibitor of oxidized cytochrome oxidase and DNP as an uncoupler of oxidative phosphorylation (Boiron *et al.* 1987; Viejo-Díaz *et al.* 2004). Uptake of $ZnPPc^{4+}$ was little affected by the presence of azide and DNP mainly when the *C. albicans* cells were preincubated with these compounds for 60 before treatment with the phthalocyanine (Fig 5). It was previously found that *C. albicans* cells treated with azide before incubation with Photofrin, during incubation with Photofrin, or both showed fluorescence equivalent to that of cells that were incubated in the absence of azide (Bliss *et al.* 2004). Therefore, it was suggested that uptake of the agent was not driven by electron transport. However, the amount of $ZnPPc^{4+}$ bound to *C. albicans* was slightly affected when the cells were incubated for a longer time (120 min) with azide and DNP before treatment with the phthalocyanine. This effect could be indicating some contribution of a mechanism of uptake dependent on energy provided via electron transport.

Photoinactivation of *C. albicans* induced by $ZnPPc^{4+}$ was analysed using different phthalocyanine concentrations and irradiation periods (Fig 6). Cells treated with 1 μM $ZnPPc^{4+}$ produced a photoinactivation of 99.99 % (~4 log decrease) after 30 min irradiation (54 J cm^{-2}). Using higher phthalocyanine concentration (>5 μM) no colony formation was detected. These results represent a value greater than 99.999 % of cellular inactivation. Moreover, this photoinactivation remained elevated during a shorter irradiation time of 15 min. When the cells were incubated with 1 μM $ZnPPc^{4+}$, an enhancement in the cell inactivation was found increasing the irradiation times. Moreover, the effectiveness of $ZnPPc^{4+}$ to photoinactivate *C. albicans* cells was evaluated after one and two washing steps (Fig 7). Under this condition, the photodynamic effect was mainly associated with $ZnPPc^{4+}$ that was tighter bound to cells. The photocytotoxic effect for cultures treated with 5 μM phthalocyanine diminished about 1 log with respect to unwashed cells, which was in agreement with a loss of the amount of cell-bound photosensitizer after one washing step. Only a minor difference in the photoinactivation of *C. albicans* was found with a successive washing step. Also, small difference was observed in the photoinactivation of cells incubated with 5 μM $ZnPPc^{4+}$ for 15 and 30 min (Fig 8). These results confirm the similar binding of the cationic phthalocyanine to cells at these incubation periods. However, an incubation time of as short as 2.5 min was sufficient to efficiently kill *C. albicans* cells (>4 log).

On the other hand, photocytotoxic effect induced by $ZnPPc^{4+}$ was studied at different cell densities (Fig 9). The high level of dependence on cell density observed with this cationic phthalocyanine reflects the strength and avidity of the binding of this photosensitizer to *C. albicans* cells. Since the majority of the $ZnPPc^{4+}$ in the incubation mixture was bound to the cells, when the cell number was increased the amount of photosensitizer bound to each cell decreases (Fig 2). This effect is mainly evidenced when the cell density increase from 10^6 to 10^8 cells mL^{-1} and therefore, the ratio $ZnPPc^{4+}$/cell diminishes tree order of magnitude. This effect is clearly confirmed in the amount of phthalocyanine bound to cells at different cellular densities. Also, opaque suspensions were mainly obtained with 10^8 cells mL^{-1}. This effect can also produce a decrease in the cell photoinactivation because light did not penetrate deep enough into the suspension to activate the photosensitizer into the cells. A higher $ZnPPc^{4+}$ concentration (50 μM) was able to produce a phototoxicity of 99.9 % in 10^7 cells mL^{-1} after 30 min irradiation. It was previously observed using pL-ce6 as photosensitizer that the effectiveness of PDI increased dramatically with decrease of cell density (Demidova & Hamblin 2005). Also, *C. albicans* were successfully treated with the result of no survival at cell densities between 10^6 and 10^8 cells mL^{-1}, when cells were irradiated with a fluence rate of 100 mW cm^{-2} for 10 min after 10 min

incubation with 6 μM ZnPcMe (Mantareva *et al.* 2007). The drug uptake dependency with an inverse behaviour to the cell density was observed for the photodynamic activity of ZnPcMe toward the fungal cells. Therefore, *C. albicans* was efficiently photoinactivated with the cationic $ZnPPc^{4+}$ using 10^6 cells mL^{-1}, as a consequence of an appropriated binding of the positively charged photosensitizer to the fungal cells. The accumulation behaviour of $ZnPPc^{4+}$ suggests that an affinity-mediated binding mechanism could be also involved in the uptake. This was proved by washing of the unbound excess of $ZnPPc^{4+}$ from the resistant *C. albicans*, indicating that the effect of fungal photoinactivation was slight decreased for all irradiation times.

Conclusions

$ZnPPc^{4+}$ was rapidly bound to the *Candida albicans* cells in a very short period of incubation. This binding to the fungal cells was very strong remaining a large amount of the cationic phthalocyanine into the cells even after two washing steps. The uptake of $ZnPPc^{4+}$ showed a marked inverse dependence on cell density. Decreasing the cell density 10-fold was accompanied by about 10-fold increase in cell uptake of $ZnPPc^{4+}$. The accumulation of $ZnPPc^{4+}$ to *C. albicans* suggests that a diffusional process was mainly involved. Photoinactivation of *C. albicans* cells was depended on the uptake of $ZnPPc^{4+}$. The effect of cell density indicates a high binding avidity of this cationic phthalocyanine to the fungal cells. The present investigation indicates that $ZnPPc^{4+}$ is an interesting photosensitizer to be applied in PDI of *C. albicans* cells.

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