Photodiagnosis and Photodynamic Therapy 13 (2016) 334–340

Contents lists available at ScienceDirect

Photodiagnosis and Photodynamic Therapy

journal homepage: www.elsevier.com/locate/pdpdt

# Photodynamic inactivation of *Candida albicans* by a tetracationic tentacle porphyrin and its analogue without intrinsic charges in presence of fluconazole

Ezequiel D. Quiroga, S. Jimena Mora, M. Gabriela Alvarez, Edgardo N. Durantini*

*Departamento de Química, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, X5804BYA Río Cuarto, Córdoba, Argentina*

ARTICLE INFO

*Article history:*
Received 28 July 2015
Received in revised form 29 August 2015
Accepted 8 October 2015
Available online 21 October 2015

*Keywords:*
Porphyrin
Photoinactivation
Antifungal
*Candida albicans*
Fluconazole

ABSTRACT

The photodynamic inactivation mediated by 5,10,15,20-tetrakis[4-(3-*N*,*N*-dimethylaminopropoxy)phenyl]porphyrin (TAPP) and 5,10,15,20-tetrakis[4-(3-*N*,*N*,*N*-trimethylaminepropoxy)phenyl]porphyrin ($TAPP^{4+}$) were compared in *Candida albicans* cells. A strong binding affinity was found between these porphyrins and the yeast cells. Photosensitized inactivation of *C. albicans* increased with both photosensitizer concentration and irradiation time. After 30 min irradiation, a high photoinactivation (~5 log) was found for *C. albicans* treated with 5 μM porphyrin. Also, the photoinactivation of yeast cells was still elevated after two washing steps. However, the photocytotoxicity decreases with an increase in the cell density from $10^6$ to $10^8$ cells/mL. The high photodynamic activity of these porphyrins was also established by growth delay experiments. This *C. albicans* strain was susceptible to fluconazole with a MIC of 1.0 μg/mL. The effect of photosensitization and the action of fluconazole were combined to eradicate *C. albicans*. After a PDI treatment with 1 μM porphyrin and 30 min irradiation, the value of MIC decreased to 0.25 μg/mL. In addition, a complete arrest in cell growth was found by combining both effects. TAPP was similarly effective to photoinactivate *C. albicans* than $TAPP^{4+}$. This porphyrin without intrinsic positive charges contains basic amino groups, which can be protonated at physiological pH. Moreover, an enhancement in the antifungal action was found using both therapies because lower doses of the agents were required to achieve cell death.

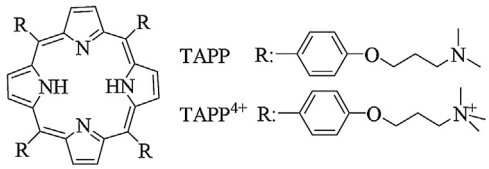


## 1. Introduction

Fungal infections have increased significantly in recent years. Now they represent an exponentially growing threat for human health due to a combination of difficult diagnosis and a shortage of effective antifungal drugs [1]. Candidiasis is an opportunist fungal infection usually caused by *Candida albicans*. This dimorphic yeast is naturally found in most humans among the normal microbiota of the skin, mucous membranes in the oral cavity, bowel and in the urogenital tract of females [2]. Because of the common eukaryotic nature of fungal and human cells, it is difficult to identify specific metabolic or structural antimicrobial targets for fungi [3]. The fundamental physiological role and the different composition of cholesterol in humans and ergosterol in fungi render the cytoplasmic membrane of *Candida* a suitable target for the action of antifungals. Triazoles are inhibitors of the cytochrome P450 14α-sterol demethylase (CYP51), an essential enzyme in the biosynthesis of ergosterol. The current treatment for candidiasis heavily relies on triazoles, even though patient responses to these antifungal drugs tend to be slow with a high risk of reinfection. Especially fluconazole has been extensively used in clinical practice due to its great efficacy and reduced toxicity. However, antifungal-resistant *C. albicans* strains have emerged with frequent exposure to fluconazole [1]. Moreover, the development of new agents in clinical therapy has lagged behind due to increasing incidence of drug resistance and few effective antifungal agents are available. Also, inadequate dosing may contribute to treatment failure and the emergence of resistance [4]. Thus, new therapies are being searched for treating fungal infections. An interesting alternative is represented by photodynamic inactivation (PDI) of microorganisms [5,6]. PDI involves the addition of a phototherapeutic agent, which is rapidly bound to cells. The aerobic irradiation of the infection with visible light produces highly reactive oxygen species (ROS), which rapidly react with a variety of substrates inducing

* Corresponding author. Fax: +54 358 4676233.
*E-mail address:* edurantini@exa.unrc.edu.ar (E.N. Durantini).

http://dx.doi.org/10.1016/j.pdpdt.2015.10.005
1572-1000/




**Scheme 1.** Structure of the TAPP and TAPP$^{4+}$ porphyrins.

damage in biomolecules. These reactions induce a loss of biological functionality leading to cell inactivation [7]. Thus, experimental investigations have demonstrated that yeasts can be effectively photoinactivated *in vitro* by several photosensitizers [8].

In recent years, efforts have been made to overcome the emergence of resistant fungi by using drug combinations [9]. Various approaches have been proposed to increase the susceptibility of *C. albicans* to fluconazole in order to cope with treatment failures. An advantage of using combinations is the additive effect, in which antifungal activity is greater than the individual contribution of each agent. Benefits can include a wider spectrum of efficacy, improved safety and a reduction in antifungal resistance. Thus, it is possible to use PDI in combination with other therapeutic approaches.

In the present work, we investigated the susceptibility of *C. albicans* to the phototoxic effect produced by 5,10,15,20-tetrakis [4-(3-*N,N*-dimethylaminopropoxy) phenyl]porphyrin (TAPP) and 5,10,15,20-tetrakis [4-(3- *N,N,N*-trimethylaminepropoxy) phenyl]porphyrin (TAPP$^{4+}$) (Scheme 1). It was previously demonstrated that TAPP$^{4+}$ is an active photosensitizer to inactivate *Escherichia coli* cells [10]. Also, we studied spectroscopic and photodynamic properties of both porphyrins in different media [11]. The photooxidation of substrates photosensitized by TAPP indicated that this intrinsically non-charged porphyrin can be an interesting agent for the photodynamic inactivation of microorganisms. Therefore, the photoinactivation activity of TAPP and TAPP$^{4+}$ were compared to establish conditions for the eradication of *C. albicans* in cell suspensions. Then, PDI mediated by these porphyrins was combined with an antifungal therapy using fluconazole to enhance both approaches.

## 2. Materials and methods

### 2.1. General

Absorption spectra were recorded on a Shimadzu UV-2401PC spectrometer (Shimadzu Corporation, Tokyo, Japan). Fluorescence measurements were performed on a Spex FluoroMax spectrofluorometer (Horiba Jobin Yvon Inc., Edison, NJ, USA). Quartz cells of 1 cm path length were used at room temperature. The cell suspensions were irradiated with a Novamat 130 AF slide projector (Braun Photo Technik, Nürnberg, Germany) equipped with a 150 W lamp. A wavelength range between 350 and 800 nm was selected by optical filters. The light was filtered through a 2.5 cm glass cuvette filled with water to absorb heat. Experiments were performed at room temperature with a fluence rate of 90 mW/cm$^2$ (Radiometer Laser Mate-Q, Coherent, Santa Clara, CA, USA). Cell growth was measured with a Turner SP-830 spectrophotometer (Dubuque, IA, USA). Chemicals from Aldrich (Milwaukee, WI, USA) were used without further purification. Fluconazole was purchased from Sigma (St. Louis, MO, USA).

### 2.2. Photosensitizers

5,10,15,20-Tetrakis[4-(3-*N,N*-dimethylaminopropoxy)phenyl]-porphyrin (TAPP) and 5,10,15,20-tetrakis[4-(3-*N,N,N*-trimethylaminepropoxy)phenyl]porphyrin (TAPP$^{4+}$) were synthetized as previously described [12]. Stock solutions (0.5 mM) of TAPP and TAPP$^{4+}$ were prepared by dissolution in 1 mL of *N,N*-dimethylformamide (DMF). The concentration was checked by spectroscopy, taking into account the value of molar absorptivity [12].

### 2.3. C. albicans *culture conditions*

The strain of *C. albicans* PC31, recovered from human skin lesion, was previously characterized and identified [13]. Yeast was grown aerobically overnight in Sabouraud (Britania, Buenos Aires, Argentina) broth (SB, 3% w/w, 4 mL) at 37 °C to stationary phase. 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), corresponding to ~$10^7$ colony forming units (CFU)/mL. The cells were appropriately diluted to obtain ~$10^6$ CFU mL$^{-1}$ or concentrated to get ~$10^8$ CFU/mL in PBS. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) were used and the photosensitizer was added from the corresponding stock solution. Cell suspensions were serially diluted with PBS and each solution was quantified by using the spread plate technique in triplicate. Viable *C. albicans* cells were monitored and the number of CFU was determined on Sabouraud agar (SA) plates after ~48 h incubation at 37 °C.

### 2.4. *Porphyrin binding to* C. albicans *cells*

Suspensions of *C. albicans* (2 mL, ~$10^6$ CFU mL$^{-1}$) in PBS were incubated in the dark at 37 °C with a set concentration (1 and 5 μM) of TAPP and TAPP$^{4+}$ for different times. The cultures were centrifuged (3000 rpm for 15 min) and resuspended with PBS. The cell pellets, after different washing steps (0 and 2) obtained by centrifugation (3000 rpm for 15 min) were resuspended in 2% aqueous sodium dodecyl sulfate (SDS) 2 mL. After that, the cells was incubated overnight at 4 °C and sonicated for 30 min. The concentration of porphyrin in the solution was measured by spectrofluorimetry (TAPP $\lambda_{exc}$ = 422 nm, $\lambda_{em}$ = 659 nm; TAPP$^{4+}$ $\lambda_{exc}$ = 420 nm, $\lambda_{em}$ = 660 nm) in a solution of 2% SDS in PBS. The fluorescence values obtained from each sample were in reference to the total number of cells contained in the suspension. The concentrations of the porphyrins in the samples were determined from a calibration curve obtained with standard solutions of the each porphyrin in 2% SDS ([porphyrin] ~0.005–0.1 μM).

### 2.5. *Photosensitized inactivation of* C. albicans *cells*

Cell suspensions of *C. albicans* (2 mL in Pyrex culture tubes of 13 × 100 mm, ~$10^6$ CFU mL$^{-1}$) in PBS were incubated with an appropriate concentration (1 and 5 μM) of porphyrin for 30 min in the dark at 37 °C. After that, the culture tubes were exposed to visible light from the bottom for different time intervals [14]. It was also studied the strength binding of the porphyrins by the cells, performing one or two washes of the suspensions, after of added the porphyrin and before illumination. Cell viability was checked as described above.

### 2.6. *Growth curves of* C. albicans

Cultures of *C. albicans* cells were grown overnight as described above. A portion (1 mL) of this culture was transferred to 20 mL of 3% w/w fresh SB medium. The suspension was homogenized and aliquots of 2 mL were incubated with 5 μM of sensitizer at 37 °C. The flasks were then irradiated with visible light at 37 °C. *C. albicans* growth was followed spectroscopically at 650 nm.

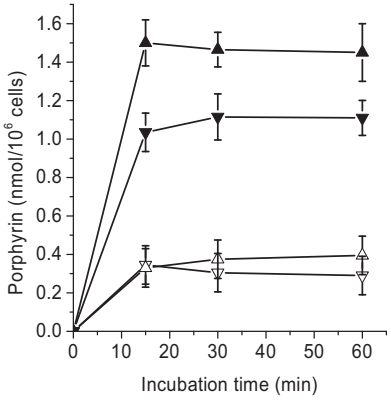



**Fig. 1.** Amount of porphyrins recovered from *C. albicans* cells (~$10^6$ CFU/mL) treated with 1 μM TAPP (▽) or TAPP$^{4+}$ (△) and 5 μM TAPP (▼) or TAPP$^{4+}$ (▲) in dark at 37 °C for different incubation times.

### 2.7. Susceptibility of C. albicans cells to fluconazole

After overnight incubation, the cells were appropriately diluted to obtain ~$10^3$ CFU/mL in 1% w/w SB medium. In all the experiments, 2 mL of the cell suspensions in Pyrex brand culture tubes (13 × 100 mm) was used, and the fluconazole was added from a stock solution 0.5 mg/mL in water. The fluconazole concentrations were tested in a range of 0.10–1.50 μg/mL. Minimum inhibitory concentration (MIC) values were determined spectroscopically at 650 nm, after 24 h of incubation. Lowest drug concentrations were determined as that promoted an inhibition of growth of ≥50% compared to the drug-free control. Combined experiments were performed incubating the cells with 1 μM porphyrin together with different concentration of fluconazole for 30 min in dark at 37 °C. After that, cell suspensions were irradiated for 30 min with visible light. MIC values were obtained as described above. The effect on growth curves of *C. albicans* was evaluated incubating the cultures (2 mL, ~$10^3$ CFU/mL) in SB medium with: (a) 5, 10 and 15 μg/mL of fluconazole, (b) the same doses of antifungal and 1 μM porphyrin, and (c) 1 μM porphyrin without fluconazole. Growth was measured as described above.

### 2.8. Controls and statistical analysis

Control experiments were performed in presence and absence of porphyrin in the dark and in the absence of porphyrin with cells irradiated. The amount of DMF (<1% v/v) used in each experiment was not toxic to *C. albicans* cells. Three values were obtained per each condition and each experiment was repeated separately three times. Differences between means were tested for significance by one-way ANOVA. Results were considered statistically significant with a confidence level of 95% ($p < 0.05$). Data were represented as the mean ± standard deviation of each group.

## 3. Results

### 3.1. Binding of porphyrins to albicans cells

The ability of TAPP and TAPP$^{4+}$ to bind to yeast cells was determined in *C. albicans* cell suspensions. Thus, cultures were incubated with 1 μM and 5 μM porphyrin for different periods. The amount of porphyrin recovered from cells after each incubation time are showed in Fig. 1. For both concentrations, TAPP and TAPP$^{4+}$ were rapidly bound to *C. albicans*, reaching the maximum binding value after 15 min incubation. For cells treated with 1 μM porphyrin, the recovered molecules were 0.30 nmol/$10^6$ and 0.38 nmol/$10^6$ cells for TAPP and TAPP$^{4+}$, respectively. When cells were incubated with

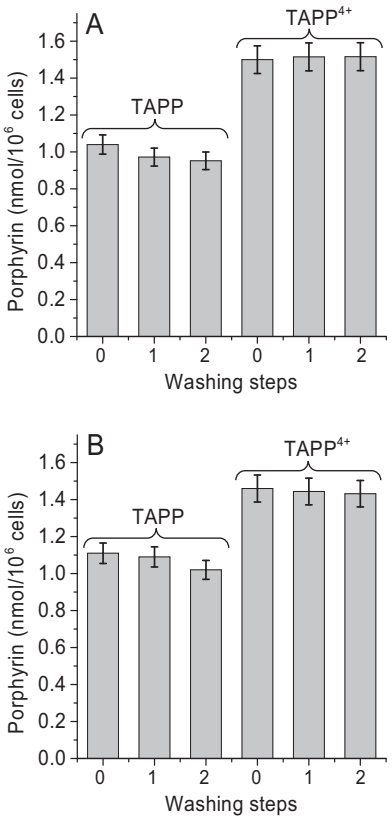



**Fig. 2.** Amount of porphyrins after different washing steps recovered from *C. albicans* cells (~$10^6$ CFU/mL) treated with 5 μM photosensitizer for (A) 15 min and (B) 30 min in dark at 37 °C.

5 μM photosensitizer, the quantity of TAPP was 1.1 nmol/$10^6$ cells, while TAPP$^{4+}$ achieved a value of 1.4 nmol/$10^6$ cells. Moreover, the amount of cell-bound porphyrin was not appreciably changed incubating the yeast cells for longer times, such as 30 and 60 min.

The binding of porphyrin to *C. albicans* was also studied after different washing steps. Thus, cultures were incubated with 5 μM porphyrin for 15 or 30 min and the cells were subjected to one or two washes with PBS. As can be observed in Fig. 2, after two washes only a small reduction was found with TAPP (<10%), while no significant differences were observed for TAPP$^{4+}$. This behavior was the same after 15 min and 30 min incubation. Similar to that observed before, the amount of TAPP$^{4+}$ recovered was slightly higher than TAPP. These results reflect the high affinity between these two photosensitizers and the *C. albicans* cells.

### 3.2. Photoinactivation of C. albicans cells

Photosensitized inactivation of *C. albicans* cells was investigated after incubation with 1 and 5 μM porphyrin. Survival curves are summarized in Fig. 3. No toxicity was found for the cells treated with both concentrations of porphyrins for 30 min in dark (result no shown). Moreover, the viability of *C. albicans* was not affected by irradiation without porphyrin. Photoinactivation of *C. albicans* was dependent on porphyrin concentrations. A linear dependence with irradiation time was observed using 1 μM porphyrin (Fig. 3), producing a reduction of 2 log in the survival after 30 min irradiation. For cells treated with 5 μM porphyrin, a fast decrease of *C. albicans* survival (~3.5 log) was detected after 5 min irradiation with visible light. An irradiation of 15 min yielded a ~4.5 log decrease in the cell viability. An increase in the irradiation to 30 min was not accompanied by an enhancement in the PDI efficiency, causing 4.4 and 4.8 log of inactivation for TAPP and TAPP$^{4+}$, respectively.

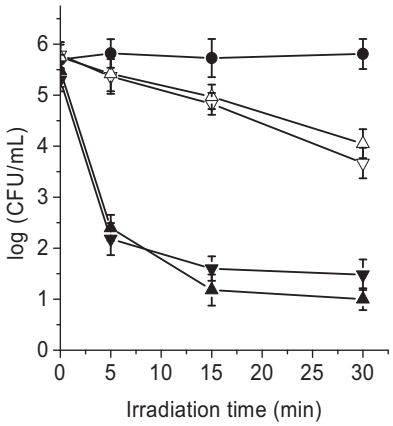



**Fig. 3.** Survival curves of *C. albicans* cells (~$10^6$ CFU/mL) treated with 1 μM TAPP (▽) or $TAPP^{4+}$ (△) and 5 μM TAPP (▼) or $TAPP^{4+}$ (▲) for 30 min at 37 °C in dark and exposed to visible light for different irradiation periods. Control cells untreated with photosensitizer and irradiated (●).

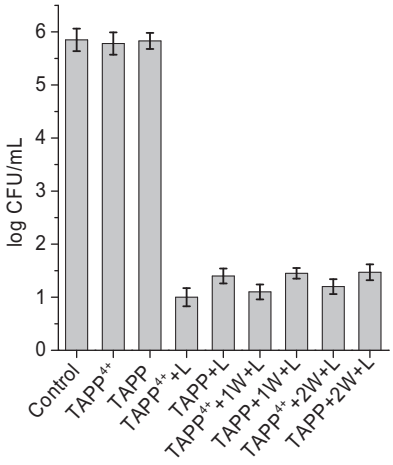



**Fig. 4.** Survival of *C. albicans* (~$10^6$ CFU/mL) incubated with 5 μM porphyrin for 30 min in dark at 37 °C and exposed to visible light for 30 min. Control: cells irradiated, L: light, 1W: one washing step, 2W: two washing steps.

Photoinactivation of *C. albicans* incubated with 5 μM porphyrin was studied after different washing steps with PBS. The survival in Fig. 4 indicates that samples washed one or two times continued responding to PDI in the same way than non-washed cells suspensions. In all cases, the cell photoinactivation showed a ~5 log reduction in the viability of yeast. There PDI activities are in agreement with the results found for porphyrin uptake because the amount of porphyrin bound to the cells was not changed by washing.

Furthermore, PDI of *C. albicans* mediated by $TAPP^{4+}$ or TAPP was examined in suspensions of different cell densities ($10^6$–$10^8$ CFU/mL). First, the binding of porphyrin to *C. albicans* was investigated treating the cell suspensions with 5 μM photosensitizer for 30 min in the dark. Fig. 5A shows the uptake of porphyrin by *C. albicans* cells using different cell densities. The amount of porphyrin bound to the fungal cells decreased with increasing of cell density. Fig. 5B shows the results of *C. albicans* photoinactivation for $10^6$–$10^8$ CFU/mL treated with 5 μM porphyrin and irradiated for 30 min. The survival increased with an enhancement in the cell density. A decrease of 2 log was found for cell suspension of $10^7$ CFU/mL incubated with $TAPP^{4+}$. Under these conditions, the cell survival of $10^8$ CFU/mL *C. albicans* was almost not changed with respect to the untreated control.

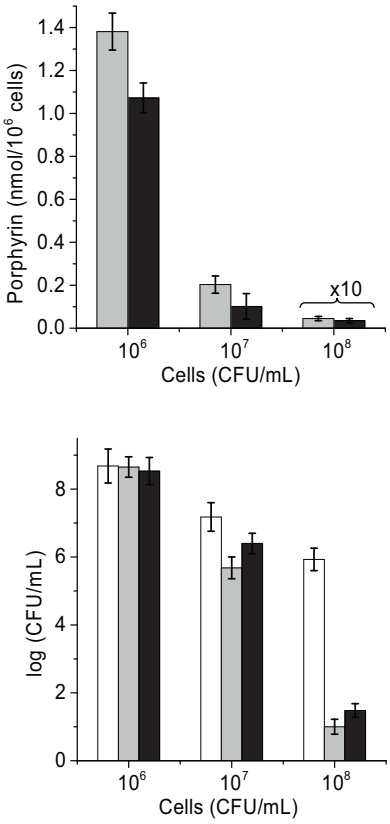



**Fig. 5.** (A) Amount of porphyrins recovered from different cell densities of *C. albicans* treated with 5 μM TAPP (black) or $TAPP^{4+}$ (grey) in dark at 37 °C for 30 min; (B) survival of *C. albicans* with different cell densities incubated with 5 μM TAPP (black) or $TAPP^{4+}$ (grey) for 30 min at 37 °C in dark and exposed to visible light for 30 min. Control culture untreated (white).

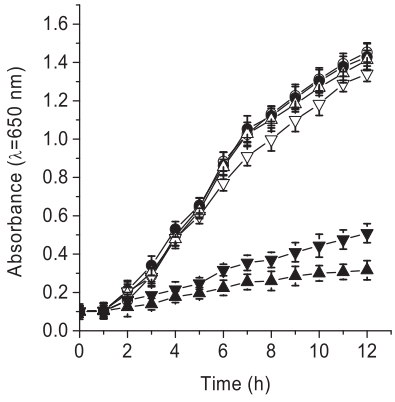



**Fig. 6.** Growth curves of *C. albicans* incubated with 5 μM TAPP (▼) or $TAPP^{4+}$ (▲) and exposed to irradiation with visible light in SB at 37 °C. Controls: untreated cells in dark (○), untreated cells irradiated (●), cells treated with 5 μM TAPP (△) or 5 μM $TAPP^{4+}$ (▽) in dark.

### 3.3. *Photosensitized effect on the growth of* albicans *cells*

The photocytotoxic activity mediated by $TAPP^{4+}$ or TAPP was investigated on growth of *C. albicans* cultures. Thus, 5 μM porphyrin was added to fresh cultures of *C. albicans* and the flasks were continuously irradiated. Fig. 6 shows the effect induced by these porphyrins on growth of cells. As can be observed, *C. albicans* cells treated with porphyrin in the dark or not treated with the photosensitizer and irradiated showed similar behavior as the controls. In contrast, growth was delayed when *C. albicans* cultures

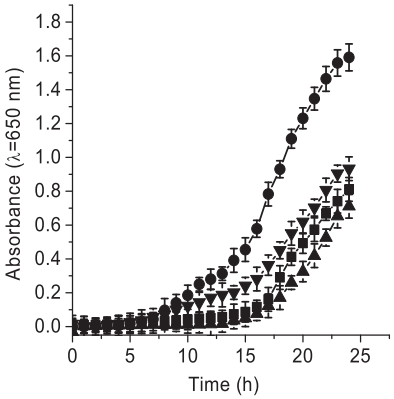



**Fig. 7.** Growth curves of *C. albicans* incubated with 5 μg/mL (▼), 10 μg/mL (■) and 15 μg/mL (▲) fluconazole and exposed to irradiation with visible light in SB at 37 °C. Controls cells (●) without fluconazole in dark.

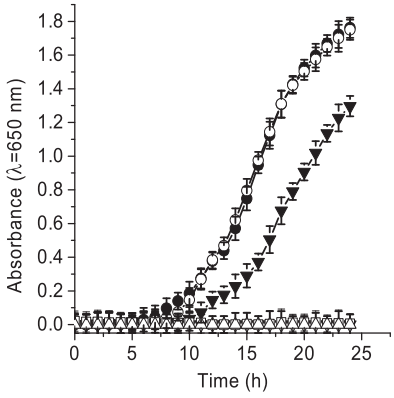



**Fig. 8.** Growth curves of *C. albicans* cells incubated with 1 μM TAPP (▼) and different concentration of fluconazole 5 (▽), 10 (□) and 15 mg/mL (△). Cultures were exposed to irradiation with visible light in SB at 37 °C. Controls: cells untreated irradiated (●) and in dark (○).

were treated with porphyrin and visible light. After irradiation in the presence of 5 μM porphyrin, the cells did not appear to be growing as measured by turbidity at 650 nm. The delay in cell growth was slightly higher for $TAPP^{4+}$ than TAPP.

### 3.4. Combined effect of fluconazole and PDI on the growth of C. albicans

The susceptibility of *C. albicans* cells to growth inhibition by fluconazole was determined in SB medium. The MIC of fluconazole was established in 1 μg/mL on this yeast. On the other hand, cells were treated with 1 μM porphyrin and irradiated for 30 min. After this sublethal porphyrin-mediated PDI, fluconazole was more active against *C. albicans*. Under this condition, the treatment with both porphyrins resulted in a reduction of the MIC to 0.25 μg/mL. Therefore, photodynamic effect improved the action of fluconazole and it was possible to obtain the same cell death at less doses of antifungal.

The effect of fluconazole was studied following the delay of the growth curve of *C. albicans*. Thus, cells were incubated with different concentration of antifungal agent. As can be observed in Fig. 7, *C. albicans* cultures incubated with fluconazole showed a growth delay compared with the control. Under this condition, *lag* phase was increased with respect to the control. The combined treatments were performed incubating the cells with different concentrations of fluconazole and 1 μM porphyrin. The results for *C. albicans* in presence of $TAPP^{4+}$ and TAPP are shown in Figs. 8 and 9, respectively. After irradiation, the cells containing fluconazole and PDI treatment no longer appeared to be growing as measured by turbidity at 650 nm. In contrast, a delay in the growth was found in the cells with only PDI approach. Therefore, cells growth stopped completely when the cultures were exposed to a combined treatment of PDI and fluconazole (Figs. 8 and 9).

## 4. Discussion

*In vitro* experiments with *C. albicans* showed that the amount of cell-bound $TAPP^{4+}$ or TAPP was not dependent on the incubation time between 15 and 60 min. A similar behavior was previously found for $TAPP^{4+}$ bound to *E. coli* cells [10]. Moreover, the uptake of $TAPP^{4+}$ and TAPP by *C. albicans* can be compared with those obtained for 5,10,15,20-tetra(4-*N*,*N*,*N*-trimethylammoniumphenyl)porphyrin ($TMAP^{4+}$) [13]. Under similar conditions, this tetracationic porphyrin reached a value of 1.48 nmol/$10^6$ cells, when the cell suspensions were incubated with 5 μM $TMAP^{4+}$. A similar value of uptake (1.70 nmol/$10^6$ cells) was found using 5,10,15,20-tetrakis(4-*N*-methylpyridyl)porphyrin ($TMPyP^{4+}$) [15]. Thus, the bindings of $TAPP^{4+}$ and TAPP to *C. albicans* were comparable to those obtained for porphyrins with cationic groups directly linked to the macrocycle. However, after two washing steps, over 90% of the initial $TAPP^{4+}$ or TAPP still remained bound to the cells, for both *C. albicans* incubated with for 15 or 30 min. In contrast, it was observed that $TMPyP^{4+}$ decreased to 30% of initial value after two washes [15]. Therefore, $TAPP^{4+}$ and TAPP persist in the cells after two washing steps indicating a strong binding affinity between these porphyrins and yeast cells. Cationic centers of $TAPP^{4+}$ are spaced from the porphyrin ring by an aliphatic chain. Thus, the positive charges have a minimal influence on photophysical properties of the porphyrin [12]. Also, the propoxy spacer provides a higher mobility of the charges facilitating the binding to microbial cells [10]. It was demonstrated that the long flexible tentacle arms of $TAPP^{4+}$ can facilitate favorable outside self-stacking interactions, while simultaneously permitting near-optimal electrostatic interaction with the DNA phosphate groups [16]. The flexible tentacle arms of the porphyrin represent a unique characteristic for an outside binder [17]. The mainly deference between $TAPP^{4+}$ and TAPP is the presence of intrinsic cationic charges in $TAPP^{4+}$ in contrast with TAPP, which is substituted by four aliphatic amine groups. These basis amine groups in the periphery of TAPP can acquire positive charges favoring a better interaction with biomembranes, depending on the medium in which the porphyrin is located [11].

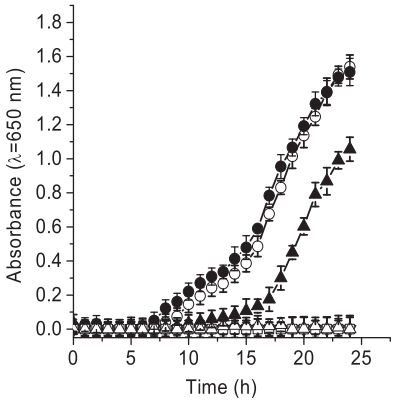



**Fig. 9.** Growth curves of *C. albicans* cells incubated with 1 μM $TAPP^{4+}$ (▲) and different concentration of fluconazole 5 (△), 10 (□) and 15 mg/mL (△). Cultures were exposed to irradiation with visible light in SB at 37 °C. Controls: cells untreated irradiated (●) and in dark (○).

Porphyrins containing cationic substituents have attracted considerable interest because of their notable ability as photother-apeutic agents. In particular, positively charged porphyrins have been proposed for the eradication of microbial infections by PDI [6,7]. Therefore, photoinactivation of *C. albicans* induced by $TAPP^{4+}$ or TAPP was analyzed using different photosensitizer concentrations and irradiation periods. When the cells were incubated with 1 μM porphyrin, an enhancement in the cell inactivation was found increasing the irradiation times. Cells treated with 1 μM porphyrin produced a photoinactivation of 99.0% (~2 log decrease) after 30 min irradiation. On the other hand, the photoinactivation remained elevated using 5 μM porphyrin even to a short irradiation time of 5 min. A value greater than 99.999% of cell inactivation was found with 15 min. These porphyrins were highly efficient in the production of $O_2(^1\Delta_g)$ with quantum yield of about 0.5 in DMF [11]. However, photodynamic activity of these porphyrins determined in homogeneous solution can be changed in the biological microenvironment. The $O_2(^1\Delta_g)$ production can decrease when the porphyrin is forming aggregates. Also, these porphyrins were efficient in producing the photooxidation of the amino acid L-tryptophan (Trp) in different systems. This amino acid can be a potential target of the photodynamic effect induced by porphyrins in cells. Moreover, Trp can be efficiently photooxidized by both type I and type II reaction mechanisms. The interaction between these porphyrins and Trp can promote an electron transfer process in the decomposition of Trp [11]. Also, $TAPP^{4+}$ was an effective photosensitizer to produce the oxidation of guanosine 5′-monophosphate and DNA photocleavage [18]. It was found a contribution of type I photoreaction processes in the DNA photodamage mediated by this cationic porphyrin. These photodynamic properties together with a high affinity for the cells make to these porphyrins efficient photosensitizing agents.

After different washing steps, the photodynamic effect was mainly associated with porphyrin that was tighter bound to cells. The photoinactivation of *C. albicans* mediated by $TAPP^{4+}$ or TAPP remained elevated even after two washing steps. These results are consistent with a small loss of the amount of cell-bound porphyrin after washing. PDI sensitized by these porphyrins were similar to those obtained for $TMAP^{4+}$ [13]. Also, $TAPP^{4+}$ and TAPP were more effective than $TMPyP^{4+}$ after 15 min irradiation [15]. A decrease in the $TMPyP^{4+}$ photosensitizing efficiency of 1 log was found when cells were washed once after 30 min irradiation. Moreover, the dependence on cell density observed with these porphyrins reveals the avidity of the binding of this photosensitizer to *C. albicans* cells. The amount of porphyrin bound to each cell decreases with an increase in the cell densities. Thus, the ratio porphyrin/cell diminishes comparably with the cell number. Moreover, the cell suspensions of $10^8$ cells/mL were opaque. This effect can also reduce the cell photoinactivation due to lower penetration of light into the cultures. PDI decreased with increasing cell density was previously observed using different photosensitizers [19,20].

Fluconazole effect on *C. albicans* was chosen since it represents one of the most commonly used azoles for treatment of candidiasis [9]. Novel approaches to fungal infections involve a conventional antifungal agent with another drug that potentiates its antifungal activity [21]. Thus, the combination of fluconazole with various non-antifungal agents is one of the strategies to cope with treatment failures. However, the effects of some agents, such as diclofenac sodium and related compounds, can reduce the sensitivity of *C. albicans* to fluconazole *via* induction of an efflux pump [22]. In the present investigation, the antifungal activity of fluconazole to *C. albicans* cells showed that yeast cells were susceptible to this compound. Also, sublethal PDI treatment of *C. albicans* produced an increase in the fluconazole susceptibility, reducing the MIC value. It was previously observed that fluconazole was more potent against *C. albicans* following methylene blue-mediated PDI, as it promoted higher inhibition of growth and a reduction of the MIC [23]. However, it was previously demonstrated that although PDI was effective against *Candida* species, fluconazole-resistant strains may be also less susceptible to photosensitization [24].

The photoinactivation mediated by $TAPP^{4+}$ and TAPP was compared in growth conditions of *C. albicans* cultures. The growth was delayed when yeast cells were treated with 5 μM porphyrin and continuously irradiated with visible light. Similar effect on the growth of *C. albicans* cultures was found using 5 μM $TMAP^{4+}$ [13]. However, 5 μM $TMPyP^{4+}$ showed a lower photodynamic activity on the growth delay [15]. Thus, to evaluate the combined effect of fluconazole and PDI on *C. albicans* growth, cell cultures were incubated with different antifungal concentrations (5–15 μg/mL). Fluconazole by itself induced a delay in the growth curve. A successful photoinactivation effect was demonstrated with the combination of fluconazole together with PDI using 1 μM porphyrin. No growth of *C. albicans* was observed combining both therapies. In the same way, the activity of a porphyrin covalently linked to an antifungal structure of fluconazole (P-fluc) was studied in *C. albicans* [25]. The results indicate that fluconazole moiety in P-fluc retained antifungal action although somewhat less than fluconazole. In addition, cytotoxicity of fluconazole in dark increased when the cultures treated with 10 μM P-fluc was irradiated due to the photodynamic effect of the tetrapyrrolic macrocycle. However, it was evidenced that covalent linkage of the antifungal fluconazole to the photosensitizing porphyrin nucleus offered no advantages over simple mixing of the two species. A promissory additive action was found using a combined treatment with 10 μM 5-(4-carboxymethylphenyl)-10,15,20-tris(4-methylphenyl) porphyrin (P-ester) and 10 μM fluconazole. However, the growth of *C. albicans* was not completely stopped because these non-cationic porphyrins were not very effective by themselves for PDI treatment of *C. albicans*. Therefore, it is necessary a combination of highly active porphyrins, such as TAPP and $TAPP^{4+}$, and a conventional antimicrobial agent to improve the activity of both therapies.

## 5. Conclusions

The porphyrins derivatives TAPP and $TAPP^{4+}$ are efficient photosensitizers to inactivate *C. albicans* cells in PBS solution. Although, TAPP is a non-charged agent, the amino groups can acquire a positive charge at physiological pH. Thus, TAPP can efficiently perform the photoinactivation of *C. albicans* cells similar to $TAPP^{4+}$. Also, the mobility of cationic groups allows a strong interaction of the photosensitizer with the yeast cells, maintaining its high activity even after washing. On the other hand, the combination of PDI treatments mediated by these porphyrins and the antifungal action of fluconazole was effective to eradicate *C. albicans*. The MIC of fluconazole decreased after a sublethal PDI treatment. Moreover, a complete suppression of cell growth was obtained by combining both effects. Thus, these porphyrins can contribute to improve the actual therapies with antifungals. The results indicate that an enhancement in the antifungal action was found using both therapies approaches because lower doses of the agents were required to achieve yeast cell inactivation.

## Acknowledgements

The authors are grateful to CONICET of Argentina (PIP 112-201101-561 00256), SECYT-UNRC (PPI 18/C400) and ANPCYT (PICT 562 0714/12) for financial support. M.G.A. and E.N.D. are Scientific Members of CONICET. E.D.Q. and S.J.M. thank CONICET for research fellowships.

## References


[1] G.J. Alangaden, Nosocomial fungal infections: epidemiology, infection control, and prevention, Infect. Dis. Clin. North Am. 25 (2011) 201–225.
[2] G. Criseo, F. Scordino, O. Romeo, Current methods for identifying clinically important cryptic *Candida* species, J. Microbiol. Methods 111 (2015) 50–56.
[3] G. Morace, F. Perdoni, E. Borghi, Antifungal drug resistance in *Candida* species, J. Glob. Antimicrob. Resist. 2 (2014) 254–259.
[4] M. Sinnollareddya, S.L. Peake, M.S. Robertsa, J. Lipmand, J.A. Roberts, Using pharmacokinetics and pharmacodynamics to optimise dosing of antifungal agents in critically ill patients: a systematic review, Int. J. Antimicrob. Agents 39 (2012) 1–10.
[5] F. Pereira Gonzales, T. Maisch, Photodynamic inactivation for controlling *Candida albicans* infections, Fungal Biol. 116 (2012) 1–10.
[6] P. Calzavara-Pinton, M.T. Rossi, R. Sala, M. Venturini, Photodynamic antifungal chemotherapy, Photochem. Photobiol. 88 (2012) 512–522.
[7] E. Alves, M.A. Faustino, M.G. Neves, A. Cunha, J. Tome, A. Almeida, An insight on bacterial cellular targets of photodynamic inactivation, Future Med. Chem. 6 (2014) 141–164.
[8] P.G. Calzavara-Pinton, M. Venturini, R. Sala, A comprehensive overview of photodynamic therapy in the treatment of superficial fungal infections of the skin, J. Photochem. Photobiol. B Biol. 78 (2005) 1–6.
[9] S. Liu, Y. Hou, X. Chen, Y. Gao, H. Li, S. Sun, Combination of fluconazole with non-antifungal agents: a promising approach to cope with resistant Candida albicans infections and insight into new antifungal agent discovery, Int. J. Antimicrob. Agents 43 (2014) 395–402.
[10] D.A. Caminos, M.B. Spesia, E.N. Durantini, Photodynamic inactivation of *Escherichia coli* by novel *meso*-substituted porphyrins by 4-(3-*N*,*N*,*N*-trimethylammoniumpropoxy) phenyl and 4-(trifluoromethyl) phenyl groups, Photochem. Photobiol. Sci. 5 (2006) 56–65.
[11] S.J. Mora, M.E. Milanesio, E.N. Durantini, Spectroscopic and photodynamic properties of 5,10,15,20-tetrakis [4-(3-*N*,*N*-dimethylaminopropoxy) phenyl]porphyrin and its tetracationic derivative in different media, J. Photochem. Photobiol. A Chem. 270 (2013) 75–84.
[12] D.A. Caminos, E.N. Durantini, Synthesis of asymmetrically meso-substituted porphyrins bearing amino groups as potential cationic photodynamic agents, J. Porphyrins Phthalocyanines 9 (2005) 334–342.
[13] M.P. Cormick, M.G. Alvarez, M. Rovera, E.N. Durantini, Photodynamic inactivation of *Candida albicans* sensitized by tri- and tetra-cationic porphyrin derivatives, Eur. J. Med. Chem. 44 (2009) 1592–1599.
[14] M.B. Spesia, M. Rovera, L. Pascual, E.N. Durantini, A new antibacterial approach using photodynamic inactivation, Chem. Educator 10 (2005) 1–4.
[15] E.D. Quiroga, M.G. Alvarez, E.N. Durantini, Susceptibility of *Candida albicans* to photodynamic action of 5,10,15,20-tetra(4-*N*-methylpyridyl) porphyrin in different media, FEMS Immunol. Med. Microbiol. 60 (2010) 123–131.
[16] L.G. Marzilli, G. Petho, M. Lin, M.S. Kim, D.W. Dixon, Tentacle porphyrins: DNA interactions, J. Am. Chem. Soc. 114 (1992) 7575–7577.
[17] N.E. Mukundan, G. Petho, D.W. Dixon, M.S. Kim, L.G. Marzilli, Interactions of an electron-rich tetracationic tentacle porphyrin with calf thymus DNA, Inorg. Chem. 33 (1994) 4676–4687.
[18] D.A. Caminos, E.N. Durantini, Interaction and photodynamic activity of cationic porphyrin derivatives bearing different patterns of charge distribution with GMP and DNA, J. Photochem. Photobiol. A Chem. 198 (2008) 274–281.
[19] T. Demidova, M. Hamblin, Effects of cell-photosensitizer binding and cell density on microbial photoinactivation, Antimicrob. Agents Chemother. 6 (2005) 2329–2335.
[20] M.A. Di Palma, M.G. Alvarez, A.L. Ochoa, M.E. Milanesio, E.N. Durantini, Optimization of cellular uptake of zinc(II) 2,9,16,23-tetrakis[4-(*N*-methylpyridyloxy)]phthalocyanine for maximal photoinactivation of *Candida albicans*, Fungal Biol. 117 (2013) 744–751.
[21] C.G. Pierce, A. Srinivasan, P. Uppuluri, K. Anand, A.K. Ramasubramanian, J.L. López-Ribot, Antifungal therapy with an emphasis on biofilms, Curr. Opin. Pharmacol. 13 (2013) 726–730.
[22] M. Urai, Y. Kaneko, M. Niki, M. Inoue, K. Tanabe, T. Umeyama, H. Fukazawa, H. Ohno, Y. Miyazaki, Potent drugs that attenuate anti-*Candida albicans* activity of fluconazole and their possible mechanisms of action, J. Infect. Chemother. 20 (2014) 612–615.
[23] I.T. Kato, R.A. Prates, C.P. Sabino, B.B. Fuchs, G.P. Tegos, E. Mylonakis, M.R. Hamblin, M.S. Ribeiro, Antimicrobial photodynamic inactivation inhibits *Candida albicans* virulence factors and reduces *in vivo* pathogenicity, Antimicrob. Agents Chemother. 57 (2013) 445–451.
[24] L.N. Dovigo, A.C. Pavarina, E. Garcia de Oliveira Mima, E.T. Giampaolo, C.E. Vergani, V.S. Bagnato, Fungicidal effect of photodynamic therapy against fluconazole-resistant *Candida albicans* and *Candida glabrata*, Mycoses 54 (2009) 123–130.
[25] S.J. Mora, M.P. Cormick, M.E. Milanesio, E.N. Durantini, The photodynamic activity of a novel porphyrin derivative bearing a fluconazole structure in different media and against *Candida albicans*, Dyes Pigm. 87 (2010) 234–240.