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Photodynamic inactivation of multiresistant bacteria (KPC) using zinc(II) phthalocyanines

Mariana Miretti [a], Romina Clementi [b], Tomas C. Tempesti [a,*], María T. Baumgartner [a,*]

[a] INFIQC (CONICET), Dpto. de Química Orgánica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Ciudad Universitaria s/n, Córdoba (5000), Córdoba, Argentina
[b] Departamento de Microbiología, Hospital Policlínico Policial de Córdoba, Córdoba (5000), Córdoba, Argentina

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ABSTRACT

The worldwide increase in antibiotic resistance has led to search of alternatives anti-microbial therapies such as photodynamic inactivation. The aim of this paper was to evaluate the photodynamic activity in vitro of a neutral and two cationic Zn phthalocyanines. Their photokilling activity was tested on *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* Carbapenemase (KPC)-producing. After treating bacteria with phthalocyanines, the cultures were irradiated with white light. As a result, the bacteria were inactivated in presence of cationic phthalocyanines. The photoinactivation was dependent of the irradiation time and phthalocyanine concentration. The most effective photosensitizer on KPC-producing was Zinc(II)tetramethyltetrapyridino[2,3-*b*:2′,3′-*g*:2″,3″-*l*:2‴,3‴-*q*]porphyrazinium methylsulfate (ZnTM2,3PyPz). After irradiation using the water soluble ZnTM2,3PyPz (3 μM) the viability of KPC (30 min of irradiation) and *E. coli* (10 min of irradiation) decreased ≈99.995%.

The antimicrobial-resistant pathogens are rapidly increasing and developing resistance to almost all available antimicrobial agents. The evolution of antimicrobial resistance is not only a natural biological phenomenon but also a result of a multitude of factors that include widespread and sometimes inappropriate use of antimicrobials. Antimicrobial resistance has become a significant public health problem.

Gram-positive and Gram-negative bacteria such as *Enterococcus faecium*, *Staphylococcus aureus*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa*, and *Enterobacter species*, are capable of resisting almost all antibiotics. Recently *Klebsiella pneumoniae* strains have received increased notoriety due to their propensity for acquiring antimicrobial resistance.[1] *K. pneumoniae* produces a number of virulence factors that contribute to pathogenesis. The capsule is considered to be the dominant virulence property and consists of an elaborate layer of surface-associated polysaccharides. The capsule and fimbriae are prominent structural components of the *K. pneumoniae* cell surface and play important roles in its survival and pathogenicity.[2]

Klebsiella pneumoniae carbapenemase (KPC)-producing are an emerging group of highly drug-resistant bacteria causing infections associated with significant morbidity and mortality. It is a frequent nosocomial pathogen, being the fourth and fifth most common cause of pneumonia and bacteriemia.[3] They efficiently hydrolyse penicillins, all cephalosporins, monobactams, carbapenems, and even β-lactamase inhibitors. Bacteria producing these enzymes are generally only susceptible to a few antibiotics, and there is high mortality among patients with bloodstream infections caused by these organisms.[4] KPC associated enterobacterial infections do not seem to be specific to sites, organs, or tissues (systemic infection). Thus, options for treating infected patients with KPC-producing are limited.[4] In 2017, the WHO included the KPC as Priority 1: CRITICAL of the priority pathogens list, with global priority to guide research, discovery, and development of new antibiotics.[5]

At present, due to resistance of bacterial strains, new antimicrobial strategies are researched to destroy bacteria with minimal invasive consequences.[6] The worldwide increase in antibiotic resistance among different classes of Gram-positive and Gram-negative bacteria has led to the research of alternative anti-microbial therapies such as photodynamic inactivation (PDI). PDI is based on the concept that a non-toxic photosensitizer can be excited by visible light of suitable wavelength to generate reactive oxygen species (ROS)[7] that react with biomolecules producing cell damage and microbial inactivation.[8] Various classes of photosensitizers have been studied.[6] The phthalocyanines (Pcs) have strong absorption in the red visible region (ca. 700 nm), low aggregation tendency, photostability and a high singlet oxygen quantum yield.[9]

* Corresponding authors.
E-mail addresses: tempesti@fcq.unc.edu.ar (T.C. Tempesti), tere@fcq.unc.edu.ar (M.T. Baumgartner).

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In recent years, phthalocyanines have shown important applications as sensitizers to PDI of multidrug resistant microorganisms, such as *Staphylococcus aureus*, *E. coli*, *Candida albicans*, influenza A virus (H1N1) and herpes simplex virus type 1.[10,11]

Cationic photosensitizers have been reported to be more effective than their anionic and neutral counterparts against Gram-negative bacteria. The cationic charges can weaken the permeability barrier of the highly organized outer membrane, allowing photosensitizers penetration into the more sensitive intracellular locations. In the photoinactivation of *E. coli* for ZnPc, cell survival after 30 min irradiation was 30%.[12]

Durantini and co-workers studied the photodynamic activity of a cationic Zn(II) tetramethyltetrapyridino[3,4] porphyrazinium salt using *E. coli*.[13] The efficiency of *E. coli* inactivation is appreciably higher for cationic than non-charged Pcs.[12] This cationic Pc produce a ~2.4 $\log_{10}$ (~99.5%) decrease of cell survival, when the cultures are treated with 10 mM of photosensitizer and irradiated for 30 min (30 mW/cm^2). While, in the study of cationic Zn(II)tetramethyltetrapyridino[2,3-*b*:2′,3′-*g*:2″,3″-*l*:2‴,3‴-*q*]porphyrazinium salt (ZnTM2,3PyPz) they reported that the photodynamic activity is higher than that previously found for ZnTM3,4PyPz under similar conditions. The main difference between ZnTM2,3PyPz and ZnTM3,4PyPz sensitizers is found in their solubilities as monomeric species in aqueous solutions.[14]

In this paper, the photodynamic activities (*in vitro*) of neutral and cationic Zn phthalocyanines were studied (Fig. 1). Their photokilling activity was tested on Gram-negatives bacteria as *E. coli* (ATCC 25922) and KPC-producing (multiresistant bacteria).

TMAZnPc[15] and ZnTM2,3PyPz[14] used in this work were synthetized[16] (see Supplementary data) and ZnPc was obtained commercially (Aldrich). The absorption spectra of TMAZnPc in DMF present a higher red-shifted λ_{max} compared to ZnPc (715 and 670 nm, respectively). In contrast, the Q-bands of ZnTM2,3PyPz in water is blue shifted by ~30 nm with respect to that of the ZnPc.[14] Regarding quantum yield of singlet oxygen $O_2(^1\Delta_g)$ production (Φ_Δ), TMAZnPc (0.40) shows lower compared to ZnPc (0.56)[17] in DMF. The Φ_Δ of ZnTM2,3PyPz was not measured due to their insufficient solubility in this solvent (see Supplementary data). In *n*-heptane/AOT (0.1 M)/water ($W_0 = 30$) reverse micelles, the Φ_Δ reported is 0.65.[14]

Phthalocyanines stock solutions were prepared in DMF (ZnPc and TMAZnPc) or water (ZnTM2,3PyPz) and stored under refrigeration in the dark.

Phthalocyanines bearing different substituents (Fig. 1) were employed to evaluate the photodynamic inactivation efficiency of both selected types of microorganisms, *E. coli* and *Klebsiella pneumoniae* carbapenemase.

The microorganisms used for this study were reference strain *E. coli* ATCC 25922 and a clinical strain provided by Hospital Policlínico Policial de Córdoba (Córdoba, Argentina) of *Klebsiella pneumoniae* carbapenemase resistant. Which is resistant to penicilins, cephalosporins of 1st, 2nd, 3rd and 4th generation, carbapenems, aztreonam, flouroquinolones, cotrimoxazole and aminoglycosides; only reserving sensitivity to fosfomycin.[18]

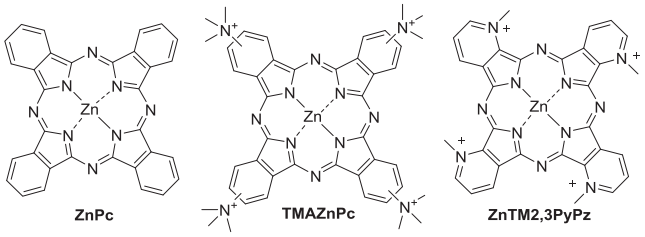

Fig. 1. Phthalocyanines structure.

In all experiments of photodynamic inactivation were used 2 mL of cell suspensions in PBS. The bacterial concentration was adjusted to 10^6 CFU/mL containing the photosensitizer at the desired concentration. For *E. coli*, 3 μM initial concentrations of Pcs were used and were increased in 3 μM until a decrease in 3 $\log_{10}$ of CFU/mL or not exceed six times initial concentration. In KPC photokilling, the final Pcs concentration was the same for *E. coli*. Then, bacteria with Pcs were incubated for 30 min in the dark at 35 °C. After that, cultures were exposed to visible light at different time intervals. The CFU was counted following 48 h incubation at 35 °C. Each experiment was carried out three independent days and the data were expressed as the average of all obtained values ± standard error media.

The ability of cationic ZnPcs to inactivate different enterobacteriaceae was compared with that obtained for unsubstituted ZnPc.

The photoinactivation efficiency was evaluated based on the decrease of the viable bacterial number ($\log_{10}$) in the tested and control samples. Significant photokilling effect was defined as ≥3 $\log_{10}$ reduction in CFU/ml.[19]

E. coli were incubated with Pcs for 30 min in the dark at 35 °C. The cultures were irradiated[20] using different visible light doses: 12.2, 18.4 and 36.9 J/cm^2, corresponding to 10, 15 and 30 min of irradiation at constant fluence rate (20.5 mW/cm^2). The viability was measured 48 h after irradiation. The survival data at different light exposure level are shown in Fig. 2a.

Control experiments, bacteria with Pcs and without light (dark control), and bacteria with light alone (light alone), were performed at 10, 15 and 30 min. Control experiments showed that

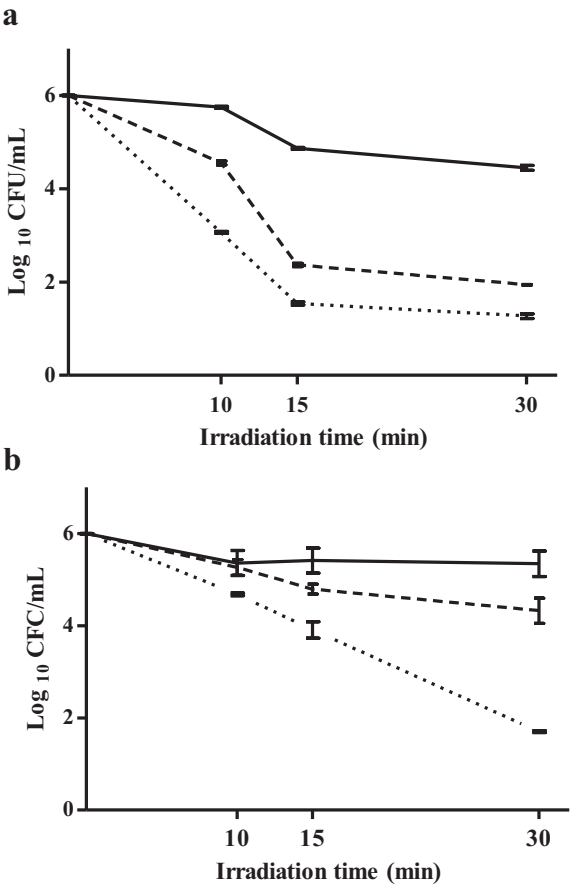

Fig. 2. Effect of the irradiation time on survival fraction a) *E. coli* (10^6 CFU/mL) after 30 min incubation with: — ZnPc (18 μM), ---- TMAZnPc (6 μM) and ····· ZnTM2,3PyPz (3 μM). b) *Klebsiella pneumoniae* multiresistant (10^6 CFU/mL) after 30 min incubation with: — ZnPc (18 μM), ---- TMAZnPc (6 μM) and ····· ZnTM2,3PyPz (3 μM).

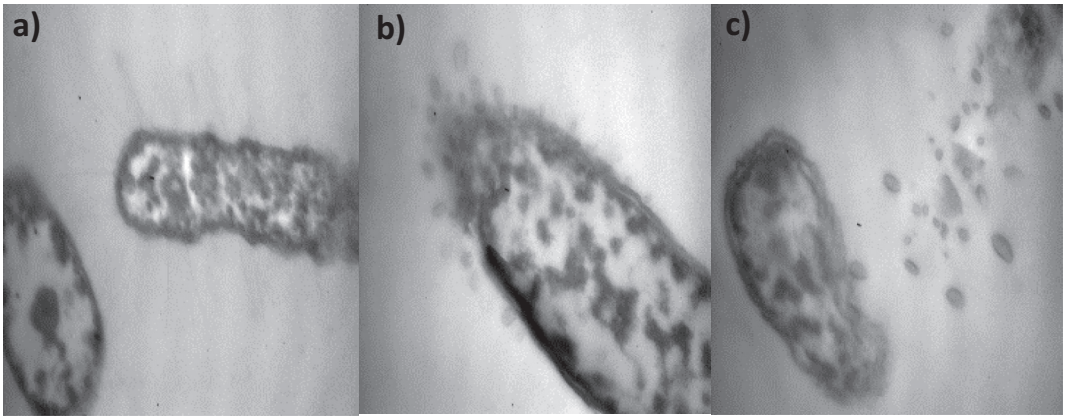

Fig. 3. TEM images (800 × 600) of KPC cells treated with 3 μM of ZnTM2,3PyPz a) without irradiation (10,000) b) and c) with 30 min irradiation (46,460).

the viability of *E. coli* after 30 min was unaffected (see supplementary information).

Results show a clear cytotoxic effect on bacteria subjected to the combination of light and either Pcs. The photoinactivation was light doses dependent. Thus, using a lower light dose (10 min) the survival fraction of *E. coli* treated is higher.

As can be observed, the photokilling activity of cationic Pcs is considerably higher than ZnPc. Thus, the photodynamic activity of ZnTM2,3PyPz produce a ≈4.7 $\log_{10}$ decrease of *E. coli* cell survival, when the cultures are irradiated for 30 min. This reduction represents ≈99.998% of cell inactivation. For this Pc, the bactericidal effect can be observed with lower light dose, only 15 min of irradiation was needed to generate 99.995% of cell inactivation. The other cationic Pc, TMAZnPc, leads to a decrease of ≈4.1 $\log_{10}$ (≈99.992% of cell inactivation) at 30 min. While under these conditions a very low diminishing in cellular viability was found using ZnPc as a sensitizer which produce a ≈1.5 $\log_{10}$ reduction representing 96.91% of cell inactivation.

An analogous behavior of neutral and cationic Pcs was previously reported.[21] The higher photodynamic activity of cationic Pcs (ZnTM2,3PyPz and TMAZnPc) respect to neutral (ZnPc) was attributed to tighter bind to Gram-negative bacteria. In these bacteria, the presence of the outer membrane, endows the surface with a negative charge. On the other hand, when the sensitizer is neutral or anionic, the photodynamic effect is lower, probably due to its cell binding failure.[12]

The difference in photoactivity between ZnTM2,3PyPz and TMAZnPc could be attributed to the different solubilities in water. TMAZnPc is slightly soluble. In addition, the values of Φ_Δ can significantly change in a different medium, diminishing mainly when the sensitizer is partially aggregated.[22] The absorption spectra of Pcs in PBS (see supplemental material) show a higher aggregation of TMAZnPc in this media. While ZnTM2,3PyPz remains the same as in water.

For PDI inhibition of *Klebsiella pneumoniae* carbapenemase cells were used ZnPc, TMAZnPc and ZnTM2,3PyPz. After 30 min of incubation with Pcs at 35 °C in dark, the cultures were irradiated for 10, 15 and 30 min. The survival data was measured 48 h after irradiation (Fig. 2b).

Control experiments showed that the viability of KPC was unaffected by 30 min of illumination alone or by 30 min with Pcs without irradiation, indicating that the cell mortality obtained after irradiation is produced by the photosensitization effect of Pcs.

The photoinactivation of KPC treated with ZnTM2,3PyPz was considerably higher than ZnPc and TMAZnPc. After 30 min irradiation using ZnTM2,3PyPz (3 μM) the viability of KPC decreased ≈99.995% (4.3 $\log_{10}$ decrease). Moreover, a very low diminishing in the cellular viability was found using TMAZnPc (6 μM) 97.48% of KPC-producing are dead (≈1.6 $\log_{10}$ decrease). ZnPc produced only 0.7 $\log_{10}$ decrease (80.05% of cellular death) with a 6-fold higher concentration than ZnTM2,3PyPz.

The difference of photokilling between *E. coli* and KPC for all Pcs may be due to presence of virulence factors in KPC producing.[2] However, ZnTM2,3PyPz produce a 4 $\log_{10}$ reduction in CFU/ml of KPC.

TEM images of KPC bacterial cells treated with 3 μM of ZnTM2,3PyPz and 30 min of irradiation are showed in Fig. 3. Some changes in the appearance of these bacteria could be observed. These results indicated that the cell envelope was significantly damaged during PDI.

The efficacy of ZnTM2,3PyPz were measured in terms of Minimal Inhibitory Concentration (MIC) and expressed in mg/L. The MIC was determined by the microdilution method using CLSI. All determinations were performed in triplicate.

The MIC of ZnTM2,3PyPz on *E. coli* was 0.008 mg/L (7.39E-3 μM) and 0.12 mg/L (1.17E-1 μM) on KPC. It is important to note that the MIC of ZnTM2,3PyPz on KPC was clinically significant since its antibacterial activities is comparable and even stronger than the available antibiotics. The KPC used in this study is only reserving sensitivity to fosfomycin (MIC $\leq$ 32 mg/L) and tigecycline (MIC = 1 mg/L),[23] and present moderate resistant to meropenem (MIC $\geq$ 8 mg/L).

In conclusion, we compare the photodynamic activity *in vitro* of a neutral (ZnPc) and two cationic (TMAZnPc and ZnTM2,3PyPz) Zn phthalocyanines. Their photodynamic activity was tested on *E. coli* and KPC. The photoinactivation was dependent of the irradiation period.

The ZnPc and TMAZnPc were moderate phototoxic against *E. coli* and KPC.

ZnTM2,3PyPz (3 μM) show effective PDI (≈99.995%) for *E. coli* and KPC after 10 and 30 min of incubation, respectively. MIC for KPC and E. *coli* were lower than other antibiotics as fosfomycin or tigecycline.

These results are promising considering the great difficulties for the treatment of multiresistant bacteria such us KPC-producing, indicating that ZnTM2,3PyPz is an interesting photosensitizer in aqueous solution to be applied in PDI of KPC-producing bacteria.

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A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2017.08.028.

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