Photoinactivation Efficiency of Cationic Porphyrin Derivatives Against Multidrug-Resistant Wound Pathogens

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Abstract

The photoinactivation efficiency of antimicrobial photodynamic therapy (aPDT) with cationic porphyrin derivatives (CPDs) against multidrug-resistant (MDR) bacterial strain was assessed. MDR bacterial strains including Pseudomonas aeruginosa, Escherichia coli, Acinetobacter baumannii, and Klebsiella pneumoniae were used. The CPDs named PM, PE, PN, and PL were synthesized as a photosensitizer (PS). A diode laser with a wavelength of 655 nm was used as a light source. Photoinactivation efficiency of the combinations formed with different energy density (50, 100, and 150 J/cm²) and PS concentrations (ranging from 3.125 µM and 600 µM) on each bacterial strain were evaluated. Toxicity of the aPDT combinations that showed a strong photoinactivation on the bacterial strains and dark toxicity of PSs and were evaluated on fibroblasts cells. In the aPDT experiments, survival reductions of up to 5.80 log₁₀ on E. coli, 5.90 log₁₀ on P. aeruginosa, 6.11 log₁₀ on K. pneumoniae and 6.78 log₁₀ on A. baumannii were obtained. There was an increase in the photoinactivation efficiency in parallel with increasing the energy density, and the best effect seen at an energy density of 150 J/cm². PL did not show any toxic effect on fibroblasts. However, other PSs were toxic in fibroblasts at high concentrations. In this research, which reflected the results of in vitro experiments, aPDT provided potent photoinactivation against MDR clinical isolates. The research results lead to an in vivo wound model study of aPDT with CPD infected with an MDR clinical isolate.

Introduction

Critical colonization or infection is a common problem in chronic wounds such as surgical site infections, burns, diabetic foot, venous leg, and pressure ulcers. Similarly, critical colonization (10⁵ colony forming units (CFU)/gram tissue) defined as the colonization of pathogenic microorganisms in the wound without clinical symptoms or pathological reactions, is an important problem frequently experienced. Critical colonization and infection on wounds retard tissue healing. The infection or critical colonization is not limited to the wound surface, progresses to deep tissues, and causes serious complications such as osteomyelitis, bacteremia, and sepsis. Source of infection on the wounds is usually polymicrobial. However, bacteria such as multidrug-resistant (MDR) Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Acinetobacter baumannii, Klebsiella pneumoniae are often isolated (Aftab et al. 2014; Braga et al. 2013; Siddiqui and Bernstein 2010).

Healing of the infected wounds is directly related to the reduction of microbial colonization on the wound. Today, antiseptics and antimicrobials are recommended to reduce the critical colonization or eliminate the infection (Nasir et al. 2016; National Pressure Ulcer Advisory Panel 2014). However, there are some problems that limit the use of these antimicrobials. Some antimicrobials are not selective to microorganisms and can damage healthy tissue. Using for more than two weeks may harm the granulation and epithelialization process. Some antimicrobials may have limited penetration to the wound tissue and biofilm layer and remain insufficient to heal the deep wound infection (Daeschlein et al. 2010; Norman et al. 2016). Therefore, the search for novel methods to accelerate wound healing has become inevitable.

Antimicrobial photodynamic therapy (aPDT) is a treatment method based on the principle of killing microbial cells using a non-toxic dye or a photosensitizer (PS) and a harmless visible light that stimulates PS. aPDT shows its antimicrobial efficacy with the reactive species formed as a result of chemical and physical reactions that occur in two different ways including Type I and Type II. The reactive species formed cause damage to bacterial DNA, cytoplasmic membrane, enzyme, and transport systems and provide antimicrobial activity (Dai, Huang, and Hamblin 2009; Hamblin and Hasan 2014; Rajesh et al. 2011). Efforts to find an ideal PS for aPDT, such as a
nontoxic, light-activated, broad-spectrum photoinactivation effect and high singlet oxygen quantum yield are ongoing. Porphyrin derivatives have the potential to be an ideal PS.

Porphyrins are macro-cyclic aromatic molecules formed by connecting four pyrrole rings together with methylene (–HC=) bridges (Nitzan and Ashkenazi 2001). Porphyrins, which originated from protoporphyrin IX (PPIX), are isolated from natural environments such as body fluids and feces of animals, eggshells, and feathers of birds. Porphyrins are of vital importance for living, including bacteria. Heme (Fe²⁺PPIX)/hemin (Fe³⁺PPIX) compound in the structure of hemoglobin and myoglobin is involved in important biological processes, such as oxygen binding, oxygen transfer, nitric oxide synthetase and transfer of electrons in cytochromes. Most bacteria meet their iron needs from Fe²⁺PPIX. Gram-positive and Gram-negative bacteria have Fe²⁺PPIX uptake mechanisms, including TomB and ExbBD proteins (Stojilkovic 2001). Natural or porphyrin derivatives using conjugates such as porphyrin-cellulose-nanocrystals or antibiotics can easily penetrate in bacteria by Fe²⁺PPIX acquisition mechanisms and demonstrate through several chemical processes such as transferring electrons, catalyzing peroxidase and oxidase reactions, absorbing photons, production of reactive oxygen species (Carpenter et al. 2012; Lippert et al. 2017).

Cationic porphyrins show their antimicrobial activity through type II reaction mechanism. Single oxygen (¹O²) formed by the transfer of the energies of light-induced cationic porphyrins to molecular oxygen reacts with structures such as phospholipids, peptides, and sterols in the cell wall and cell membrane and cause cell death (Alves et al. 2014; Amos-Tautua, Songca, and Oluwafemi 2019; Reddi et al. 2002; Tavares et al. 2011). This result has been confirmed in the studies using CPDs such as T₄MPyP (Reddi et al. 2002), 5,10,15,20-tetakis (1-methyl-pyridino)-21H, 23H-porphine, tetra-p-tosylate salt (TMP) (Collins et al. 2010), Tetra-Py+Me, Tri-SPy+Me-PF (Alves et al. 2013), meso-tetra(pyren- 1-yl)porphyrin (TPyP), metallo-tetrapyrenylporphyrins NiTPyP, CuTPyP and ZnTPyP compounds (Zoltan et al. 2010), tetracationic zinc(II) tetrapyridinoporphyrazine (Ragás et al. 2013), mesoimidazolium-substituted porphyrin derivative (ImP) and pyridinium-substituted porphyrin derivative (PyP) (Prasanth et al. 2014), 5,10,15,20-tetakis(4-nitrophenyl)porphyrin (TNPP) and zinc porphyrin complex (ZnTNPP) (Rahimi et al. 2016), porphyrin – magainin conjugate and porphyrin – buforin conjugate (Dosselli et al. 2014). In this current study, different from previous studies, the aim was to obtain a broad spectrum photoinactivation efficiency on MDR clinical isolates with the CPDs synthesized by us. In our previous study, a strong photoinactivation was obtained on methicillin-resistant Staphylococcus aureus (MRSA) (Taslı et al. 2018). The goals in this present study in which multi-drug resistant Gram negative bacteria were used can be listed as follows:

- To determine the light energy density and PS concentration ranges that create photoinactivation on the strain selected representing each species;
- To investigate the photoinactivation of the combinations of energy density and PS concentration providing strong photoinactivation on other strains of each species.
- To investigate the toxicity of all aPDT combinations with photoinactivation effect on fibroblasts.

**Methods**

**Bacteria**

A total of 21 clinical isolates including *P. aeruginosa* (n = 3), *E. coli* (n = 2), *A. baumannii* (n = 14), *K. pneumoniae* (n = 2) were used. These clinical isolates with MDR were obtained from Izmir Katip Çelebi University Faculty of Medicine, Microbiology Laboratory. Antibiotic susceptibilities of bacteria using routine tests according to Clinical...
Laboratory Standards Institute-2018 (CLSI-2018) recommendations were evaluated (Institute 2018), and the results was as shown in supplementary 1.

Photosensitizer

CPDs named PM, PE, PN and, PL were synthesized by researchers at Ege University Faculty of Pharmacy Pharmaceutical Chemistry Department. The chemical structure and absorbance values of the CPDs which previously evaluated for aPDT efficacy on MRSA by researchers (Taslı et al. 2018) were shown in Fig. 1a. CPDs can absorb a wavelength in the broad spectrum varying from 250 to 800 nm and, the maximum light absorption was at 422 ± 3 nm (Fig. 1b).

Chemistry: The infrared (IR) spectra of the compounds were monitored by attenuated total reflectance (ATR) (PerkinElmer Spectrum 100 FT-IR, Waltham, MA, USA). \(^1\)H NMR spectra were recorded on an Agilent 600 MHz Premium COMPACT NMR spectrometer (Santa Clara, CA, USA) by using tetramethylsilane (TMS) as an internal standard and DMSO-d\(_6\) as a solvent. Chemical shifts were measured in parts per million (δ). The \(J\) values were given in Hz. Abbreviations for data quoted are: s, singlet; d, doublet; t, triplet; q, quartet; quin, quintet; sextet. All reagents and solvents were of reagent-grade quality and obtained from commercial suppliers (Sigma, Acros and Merck). Elemental analyses (C, H, N) were performed by Leco TruSpec Micro (Leco, St. Joseph, MI, USA).

General procedure for the synthesis of the final compounds: CPDs were prepared according to the method of Gomes et al., 2011 (M. Gomes et al. 2011). Excess of the corresponding alkyl halide (iodomethane, ethyl bromide, propyl bromide, 3-Bromo-1-propanol) (65 mmol) was added to a suspension of 5,10,15,20-tetrakis(4-pyridyl)porphyrin (120 mg, 193.9 µmol) in DMF (30 mL). After refluxing for 1–8 h, the mixture was cooled to room temperature and the obtained precipitate was filtered and washed with diethyl ether or ethanol. The crude product was taken in acetone-water (1:1) and then filtered, washed with acetone. The structures of the final compounds were confirmed by FT-IR, \(^1\)H NMR, and elemental analysis. The purity levels of compounds were determined by elemental analysis (C, H, N), and the results were within ± 0.4% of the calculated values. Spectral and elemental analysis data of the compounds were reported in the supporting information (Supplementary 2).

Minimal inhibitory concentrations (MIC) of the CPDs for \(E.\)coli-1, \(P.\) aeruginosa-1, \(K.\) pneumoniae-1, and \(A.\) baumannii-1 were evaluated by the micro-dilution method according to CLSI-2018 to determine antimicrobial activity and predict maximal PS concentration levels that could be used in photoinactivation experiments (Institute 2018). The MIC experiments evaluated at Ege University Pharmaceutical Microbiology Laboratory was done as described in the study (Taslı et al. 2018). 1 mL of Tryptic Soy Broth (TSB) containing bacteria was centrifuged (3000 rpm at 4 ° C for 10 minutes) and the supernatant was discharged without touching the pellet. 1 ml of phosphate buffer saline (PBS) was added onto the pellet remaining at the bottom and suspended. Bacterial suspensions were adjusted to standard McFarland 0.5 (1.5×10^8 CFU/mL) turbidity and further diluted to give a final inoculum size of 5×10^5 CFU/mL per well. 50 µL of Mueller Hinton Broth (MHB) was transferred to each well of 96 well plates. The two-fold serial dilutions were made by adding 50 µL PS dissolved in PBS/antibiotic to the first wells of the plates. In the evaluation, ciprofloxacin was adjusted from an initial concentration of 16 µg/mL to a final concentration of 0.016 µg/mL and, PSs were adjusted from an initial concentration of 5.000 µg/mL and final concentrations of 2.441 µg/mL. 50 µL of bacteria suspension (5×10^5 CFU/mL) was added to each well of the plates and, the plates were allowed to incubate at 37°C for 16–18 h. After the incubation, the MIC values of the CPDs were determined by calculating the lowest compound concentrations that prevent the growth of bacteria. All experiments were performed in triplicate, each being repeated at least three times.
Laser device and optical set up

A diode laser with a wavelength of 655 nm was used as a light source (PSU III.LED; Changchun New Industries Optoelectronics Co. Ltd., Changchun, China). The distance between the optical plate and the fiber tip was 8.7 cm, and the light illuminated an area of 3.14 cm$^2$ on the optical plate from this distance. The output power of light was measured as 41.5 milliwatts (mW) at the plate surface.

Photoinactivation experiments

Photoinactivation experiments were carried out in two stages at Izmir Katip Çelebi University Central Research Laboratories Biomedical Optics and Laser Applications Laboratory. Firstly, bacterial photoinactivation evaluation was carried out by using combinations of different energy density (J/cm$^2$) and PS concentrations (μM) on a single clinical isolate representing each species. Because clinical isolates of each species had a similar antibiotic resistance profile (Supplementary 1), randomization was used to select representative isolates. In these pioneering experiments, the photoinactivation efficiency of aPDT combinations on representative clinical isolates was determined. The combinations for each PS were determined as follows: aPDT experiments were started with low energy density and PS concentration. The energy density was kept constant and the PS concentration gradually increased until a strong antimicrobial activity was achieved. After this stage, the energy density was increased and the PS concentrations were gradually reduced until limited antimicrobial efficacy was developed. In determining the maximum PS concentration that can be used in the combinations, the MIC values of PSs were taken into account. In the second step, the photoinactivation effect on the other strains of the bacteria species was evaluated for the combination that had a strong photoinactivation on the selected bacterial strain. The following groups were formed in each of the aPDT experiments:

- Laser group (L) where light was applied alone,
- PS group where PS was applied alone,
- aPDT group where both the light and PS were applied together,
- Control group (C) where PBS was applied alone.

50 μL of the bacterial suspension that adjusted to standard McFarland 0.5 (1.5×10$^8$ CFU/mL) turbidity was transferred to specific wells of each of 96 well plates identified as PS, aPDT, L, and C. 50 μL PS from the stock suspensions at specific concentrations was added to wells of the PS and aPDT group plates containing bacteria. 50 μL of PBS was added to the wells of the L and C group plates with bacteria. All four groups were incubated for 15 min at room temperature. The aPDT and L group plates were exposed to light. After light exposure, bacterial suspensions in all groups were diluted by serial dilution using PBS. 100 μL of aliquot was taken from the dilutions and plated on tryptic soy agar and left for an overnight incubation in the dark area at 37 °C. After incubation, bacterial survival was calculated at CFU/mL. Each experiment was repeated three times in triplicate.

Toxicity on the fibroblast cells

These experiments were carried out as described in study (Topaloğlu et al. 2020) at Izmir Katip Çelebi University Central Research Laboratories Biomedical Optics and Laser Applications Laboratory. A healthy mouse skin
fibroblast cell line (L929) was used in the study. The cells were cultured in 75 cm² tissue culture flasks in Dulbecco's Modified Eagle's Medium (DMEM) (Sigma Aldrich, Germany) solution, containing 1% L Glutamine (Gibco, USA), 10% Fetal Bovine Serum (FBS) (Gibco, USA) solution, and 1% Penicillin/Streptomycin (Gibco, USA). These cells were incubated in a humidified environment containing 95% air and 5% CO² until they form a confluent culture in a single layer. The cells reaching 80% confluence were washed with PBS and trypsinized using 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) (Biological Industries, Israel). 2×10⁴ fibroblast cells were seeded into each well of 96-well plates and allowed to incubate at 37°C for 24 h so that the cells adhere to the wells of the plate. Then the cell culture medium was discarded and the experimental process continued as described in the groups below.

The dark toxicity of the concentrations ranging from 25 to 600 µM for PM, 3.125 to 400 µM for PE, PN, and PL on the fibroblast was performed using 15 min and 24 h incubation. In the experiment, 100 µL PS suspension from stock suspension dissolved at specific concentrations in cell culture medium for dark toxicity groups was transferred to plate wells containing fibroblasts. 100 µL cell culture medium without PS was transferred to control group plate wells. The plates wrapped with aluminum foil to create a dark environment and allowed to incubate at 37°C incubate for 15 min or 24 h.

The toxicity of aPDT on the fibroblast cells was performed using 15 min and 24 h incubation. 100 µL from PS suspension dissolved at specific concentrations in cell culture medium was transferred to plate wells containing fibroblasts cells (Combinations were as in Fig. 7b). 100 ml cell culture medium without PS was placed in the control and light group plate wells containing fibroblast cells. The plates wrapped with aluminum foil to create a dark environment and allowed to incubate at 37°C incubate for 15 min. Phototoxicity or L (50 J/cm², 100 J/cm², and 150 J/cm²) and aPDT groups were irradiated at an appropriate energy density with a diode laser. Control and PS groups were taken from the incubator but not exposed to ambient light or laser. After the light application to aPDT or light groups was finished, all the plates wrapped with aluminum foil again and allowed to incubate at 37°C incubate for 15 min or 24 h.

Following, cell culture medium or PSs added to the plate wells were removed. Cells in the wells were washed with PBS. 100 µL MTT (4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide (5 mg/mL) (Sigma, St. Louis, MO, USA) was added to each well. After 2 h of incubation, the formazan crystals were dissolved with 100 µL of DMSO, and the absorbance was measured at 570 nm with a microplate reader (iMark, Bio-Rad Lab., USA.). The absorbance values were used to determine the change in survival in fibroblast cells. Control groups were used for each experiment. Each experiment was repeated three times in triplicate.

Data analysis and Evaluation

In the photoinactivation experiments, the calculations were done as described below: First, bacterial survival in CFU/mL for each plate was calculated according to formula 1.

Formula 1

\[
\text{CFU/mL} = \text{Number of colonies per mL on the plate} \times \text{Total dilution factor}
\]

The control group was taken as a reference for determining survival reduction of the aPDT, PS, or L groups. Survival reductions were calculated as logarithmic as shown in formula 2.
For the toxicity on the fibroblast cells, the calculations were done as follows. The control group was taken as a reference for determining the toxic effect of dark toxicity, phototoxicity, or aPDT applications on fibroblast cells. Percentage changes of the fibroblast cell survival based on the absorbance values of the groups were calculated according to formula 3.

**Formula 3**

\[
\text{Cellviability(\%)} = \frac{(\text{Absorbance value of control group} - \text{Absorbance value of the application group}) \times 100}{\text{Absorbance value of the control group}}
\]

SPSS 16.0 was used for data analysis. "Paired sample t-test" was used to determine the differences of laser or aPDT groups compared to the control. In the comparisons, differences with \( p < 0.05 \) were accepted as statistically significant.

**Results**

**Antibacterial activity**

The MIC values of CPDs and ciprofloxacin for bacterial strains ranged from 850.40 to > 7365.51 \( \mu \text{M} \) (Table 1).

**Photoinactivation of the bacterial strains**

In the experiments, the photoinactivation efficiency of the combinations (energy density and PS concentration) on the clinical isolates representing the species for each PS was determined. In these preliminary experiments, the combinations with low PS concentration that provided strong photoinactivation on the clinical isolates were selected. In secondary experiments, the photoinactivation efficiency of combination selected by pioneering experiments was examined on other clinical isolates of the species.

Figure 2a shows the photoinactivation efficiency of the aPDT combinations on \( E. \text{coli-1} \). A strong photoinactivation was not seen at any of the PM concentrations combined with 50 J/cm\(^2\). For the other three PSs, [50 J/cm\(^2\)-400 \( \mu \text{M} \)] combination created a strong photoinactivation. For all four PSs, [150 J/cm\(^2\)-200 \( \mu \text{M} \)] combination provided a survival reduction ranging from 4.73 to 5.71 log\(_{10}\). The same combination as seen in Fig. 2a showed strong photoinactivation effect on \( E. \text{coli-2} \). The survival reductions in the aPDT groups using PM, PE, PN, and PL were 3.10 log\(_{10}\), 4.72 log\(_{10}\), 5.16 log\(_{10}\), and 5.04 log\(_{10}\), respectively.

Figure 3 shows the photoinactivation results of the aPDT combinations on \( P. \text{aeruginosa} \). The combinations that produced strong photoinactivation on \( P. \text{aeruginosa-1} \) were [100 J/cm\(^2\)-600 \( \mu \text{M} \)] for PM, [100 J/cm\(^2\)-300 \( \mu \text{M} \)] for PE and PN, and [100 J/cm\(^2\)-100 \( \mu \text{M} \)] for PL. These combinations created survival reductions ranging from 4.07 to 5.02 log\(_{10}\) (Fig. 3a). As shown in Fig. 3b, [100 J/cm\(^2\)-600 \( \mu \text{M} \)] combination using PM provided a reduction ranging from
3.82 to 5.90 log in other three P. aeruginosa isolates. For both PE and PN, the survival reduction in all three clinical isolates ranged from 4.99 to 5.82 log at [100 J/cm²-300 µM] combination. For PL, reduction of P. aeruginosa isolates reached 5.04 log at [100 J/cm²-100 µM] combination.

For K. pneumoniae-1, the combinations with strong photoinactivation for PM, PE, PN, and PL were [150 J/cm²-600 µM], [150 J/cm²-100 µM], [150 J/cm²-200 µM], and [150 J/cm²-100 µM], respectively (Fig. 4a). For all four PSs, these same combinations provided reductions ranging from 3.92 to 6.11 log in K. pneumoniae-2 (Fig. 4b).

In the preliminary experiments with A. baumannii-1, there was strong photoinactivation at [100 J/cm²-100 µM] combination for PM and [50 J/cm²-50 µM] combination for other the three PSs (Fig. 5a). [100 J/cm²-100 µM] combination using PM provided reductions ranging from 4.43 to 6.58 log in other clinical isolates except for A. baumannii-8 (3.57 log) and A. baumannii-10 (3.83 log). Survival reduction provided by [50 J/cm²-50 µM] combination using PE, PN, and PL varied from 4.06 to 6.78 log in other clinical isolates except for A. baumannii-10 (PN, 3.76 log) (Fig. 5b).

Toxicity on the fibroblast cells

The toxicity of the concentrations for each PS used on clinical isolates was investigated on fibroblast cells. Any of PM concentrations at 15 min incubation did not show dark toxic effects on fibroblast cells. 200 µM and above of PM concentration at 24 h incubation resulted in a decrease in survival ranging from 9.52 to 41.99% (Fig. 6a). PE, which did not show dark toxic effects after 15 min of incubation, caused reductions varying between 18.15 and 32.70% at 24 h incubation (Fig. 6b). PN was toxic at 50 µM and above concentrations at 15 min of incubation. All PN concentrations were toxic at 24 h incubation, and the survival decline ranged from 18.5 to 57.80% (Fig. 6c). At the 15 min of incubation, the toxic effect for PL began at concentrations above 200 µM. It caused fibroblast cell reductions ranging from 15.38 to 31.95% at 24 h incubation (Fig. 6c).

Figure 7 shows the efficacy of the aPDT and light on fibroblast cells. [50 j/cm²-100µM] combination using PM provided 31.43% and 13.65% reduction in the fibroblasts at 15 min and 24 h incubation, respectively. In other combinations, there was cell reduction at 15 min of incubation, while cell proliferation varying from 58.96 to 94.82% occurred at 24 h incubation (Fig. 7a). For PE and PN, [50 j/cm²-50µM] at both incubation times did not cause a significant reduction in fibroblast cells compared to other combinations (Fig. 7b and Fig. 7c). There was no significant reduction in any combination of fibroblast cells at both 15 min and 24 h incubations for PL (Fig. 7d).

No phototoxicity was observed at any of the incubation periods. On the contrary, proliferation was seen and significant, especially at 100 J/cm² and 150 J/cm².

**Discussions**

In the aPDT application, it is among the primary priorities that the PS can only be activated by light, has a high wavelength absorption capacity, and a maximal level of antimicrobial activity in a wide spectrum (Klausen, Ucuncu, and Bradley 2020; Taslı et al. 2018). In this current study, MIC evaluation results showed that the antimicrobial activities of the CPDs without light were negligible. CPDs provided a strong antimicrobial activity against MDR clinical isolates at concentrations well below the MIC values. With this feature, CPDs met one of the important conditions to be a PS.
In pioneering photoinactivation experiments on clinical isolates, PS concentrations ranging from 50–600 µM were required for 3 log and above bacterial reduction. On the other hand, 300 µM and above for PM and 200 µM and above for PE, PN, and PL at 24 h incubation caused dark toxicity on the fibroblast cells. This result will limit the use of CPD concentrations of 200 and above in aPDT combinations. However, the finding of increased dark toxicity due to increased CPD concentration was consistent with the literature (Hanakova, Bogdanova, Tomankova, Pizova, et al. 2014; Jimenez-Banzo et al. 2008; Lambrechts et al. 2005; Yuan et al. 2017).

Combinations of aPDT with high PE and PN concentrations and energy density that provided photoinactivation on the clinical isolates resulted in a reduction in survival of up to 50% in fibroblast cells. However, limited photoinactivation reduction (below 13%) at [50 J/cm²-50 µM] combination for PE and PN may be promising. On the other hand, the photoinactivation efficiency of the combination [50 J/cm²-50 µM] against clinical isolates was very low. Contrary to dark toxicity experiments (Fig. 6a and Fig. 6d), at 24 h incubation, aPDT combinations containing concentrations of 200 µM for PL, 300 µM and above for PM did not damage fibroblast cells (Fig. 7a and Fig. 7d). The reason for these discordant results may be related to the proliferation effect of light on fibroblast cells. Thus, a significant increase on fibroblast cells was observed at 100 J/cm² and 150 J/cm² energy density (Fig. 7e). These results were in line with the results that red light induces cell proliferation by increasing the release of essential fibroblast growth factors (Cios et al. 2021; Fortuna et al. 2018; George, Hamblin, and Abrahamse 2018; Kim et al. 2019; Li et al. 2017; Niu et al. 2015; Sperandio et al. 2015; Zhang et al. 2003). However, attributing fibroblast cell proliferation only to the biostimulant effect of light in aPDT application may be an incomplete approach. aPDT application may also have contributed to fibroblast proliferation. However, no research has been found to support or refute this assumption. On the other hand, in most of the research has been reported that the toxic effects of cationic porphyrins on fibroblast cells increase in parallel with the increase in concentration and energy density (Dosselli et al. 2014; Hanakova, Bogdanova, Tomankova, Binder, et al. 2014; Lambrechts et al. 2005; Liu et al. 2012). There were studies in which a strong photoinactivation effect was obtained at PS concentrations much lower than the PS concentrations used in the current study. However, these studies evaluated the efficacy of aPDT against wild-type bacteria (Alenezi et al. 2017; Almeida et al. 2014; Alves et al. 2013; Banfi et al. 2006; Collins et al. 2010; Dosselli et al. 2014; M. C. Gomes et al. 2013; Liu et al. 2012; Maisch et al. 2012; Skwor et al. 2016; Tavares et al. 2010; Tomé et al. 2004) using a blue light source (Banfi et al. 2006; Fila, Kawiak, and Grinholc 2016; Liu et al. 2012; Nitzan and Ashkenazi 2001). In the current study, the requirement for high concentration and energy density for strong photostimulants may be related to the light source used. Red light has more tissue penetration than blue light (Nitzan and Ashkenazi 2001; Tasli et al. 2018). Therefore, the red light was used for CPDs with absorbance values in the range of 250–800 nm (wavelength average: 422 ± 3 nm). In this current study, the photoinactivation efficacy provided by aPDT combinations in all clinical isolates of each strain was consistent. However, this generalization did not apply to PM. In the aPDT experiments using PM, photoinactivation efficiency decreased between 22.94% and 32.24% in some other clinical isolates of the strain. Interestingly, unlike other bacterial species, low energy density and PS concentration provided a strong photoinactivation effect in MDR A. baumannii strains. Further analysis is needed to explain this finding, such as photosensitizing absorption, measurement of ROS, and O₂ formation amounts.

The current research was aimed to determine whether CPDs are ideal PS by pioneering experiments. In particular, PN and PE are not suitable for aPDT at high concentrations and energy density without undergoing modifications that inhibit host cell entry. However, the exciting results regarding PL were promising. Compared with other PSs, aPDTs using PL (5,10,15,20 Tetrakis(N-(3-hydroxypropyl)pyridinium-4yl)porphyrin tetrabromide) had little or no
toxic effects on fibroblast cells. The presence of the hydroxyl group may have made this difference. Our next step will be to investigate these reasons that make the difference for PSs.

**Declarations**

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**Conflict of Interest**

No conflict of interest declared should appear within this section.

**Appendix A. Supplementary data**

Supplementary data to this article can be found on line at ......

**Author Contribution**

Research design: Ayşe AKBIYIK, Hüseyin TAŞLI, Nermin TOPALOĞLU

Synthesis of CPDs: Vildan ALPTÜZÜN, Sülünay PARLAR

Identification and investigation of antibiotic susceptibility of clinical isolates: Selçuk KAYA

Evaluation of minimum inhibitory concentrations of CPDs: Ayşe AKBIYIK, Hüseyin TAŞLI

Photoinactivation experiments: Ayşe AKBIYIK, Hüseyin TAŞLI, Nermin TOPALOĞLU

Evaluation of fibroblast toxicity: Ayşe AKBIYIK, Hüseyin TAŞLI, Nermin TOPALOĞLU

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Table

Table 1. Minimal inhibitory concentrations of CPDs and ciprofloxacin

| CPDs and concentration values | E. coli-1 | P. aeruginosa | K. pneumoniae | A. baumannii |
|------------------------------|-----------|---------------|---------------|-------------|
| PM  | µg/mL     | 1250         | >5000         | 5000         | 2500        |
|     | µM        | 1841.38      | >7365.51      | 7365.51      | 3682.75     |
| PE  | µg/mL     | 625          | 5000          | 5000         | 5000        |
|     | µM        | 850.40       | 6803.18       | 6803.18      | 6803.18     |
| PN  | µg/mL     | 1250         | 1250          | >5000        | 2500        |
|     | µM        | 1580.16      | 1580.16       | 6320.64      | 3160.32     |
| PL  | µg/mL     | 5000         | 5000          | 2500         | 1250        |
|     | µM        | 5847.61      | 5847.61       | 2923.81      | 1461.90     |
| Ciprofloxacin*               | µg/mL     | 2            | >16           | >16          | >16         |

*Acceptable quality control ranges of minimum inhibitory concentrations of ciprofloxacin for reference strains.

Figures
Figure 1

Features of CPDs. a. The chemical structures of CPDs. b. Light absorptions of the CPDs. (Perkin Elmer UV Winlab Data and Viewer Version 1.1.00).
**Figure 2**

The photoinactivation results for MDR E. coli. a. Reduction of MDR E.coli-1 survival at the combinations formed by different energy density and PS concentrations b. The photoinactivation results of the selected combinations at 150 J/cm² for MDR E.coli-2.
### Figure 3

The photoinactivation results for MDR *P.aeruginosa*. Reduction of MDR *P.aeruginosa-1* survival at the combinations formed by different energy density and PS concentrations. The photoinactivation results of the selected combinations at 100 J/cm² for other MDR *P.aeruginosa* isolates.
Figure 4

The photoinactivation results for MDR K.pneumoniae. Reduction of MDR K.pneumoniae-1 survival at the combinations formed by different energy density and PS concentrations b. The photoinactivation results of the selected combinations at 100 J/cm² for MDR K.pneumoniae-2.
Figure 5

The photoinactivation results for MDR A.baumannii. Reduction of MDR A.baumannii-1 survival at the combinations formed by different energy density and PS concentrations b. The photoinactivation results of the selected combinations at 50 J/cm² for other MDR A.baumannii isolates.
Figure 6

Dark toxicity on the fibroblast cells. a. Cell viability at 25µM-600 µM PM concentrations. b. Cell viability at 3.125 µM-400 µM PE concentrations. c. Cell viability at 3.125 µM-400 µM PN concentrations. d. Cell viability at 3.125 µM-400 µM PL concentrations (*p < 0.01, **p < 0.001, significant difference versus the control group).
Figure 7

Cytotoxicity and phototoxicty of the aPDT combinations on fibroblast cells. a. Cell viability at aPDT combinations using PM. b. Cell viability at aPDT combinations using PE. c. Cell viability at aPDT combinations using PL. d. Cell viability at aPDT combinations using PN. e. Cell viability at an energy density of 50 J/cm², 100 J/cm² and 150 J/cm² (*p < 0.01, **p < 0.001, significant difference versus the control group).

Supplementary Files

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