Scientific paper

Photoinactivation of MRSA ATCC 33591 by antimicrobial photodynamic therapy using coumarin-functionalized zinc(II) phthalocyanine

Merve Bilgin,[1] Tugba Elgun,[2,3] Cigdem Aktas,[4] Onur O. Karagulle,[5] Kamil Çınar,[6] Asiye Gok Yurttas[7,*]

[1] *Department of Pharmaceutical Microbiology, Faculty of Pharmacy, Istanbul Health and Technology University, Istanbul, Türkiye*

[2] *Medical Biology, Faculty of Medicine, Biruni University, Istanbul, Türkiye*

[3] *Biruni University Research Center (B@MER), Biruni University, 34015, Istanbul, Türkiye*

[4] *Department of Genetics, Aziz Sancar Institute, Istanbul University, Istanbul, Türkiye*

[5] *Department of General Surgery, Istanbul Training and Research Hospital, University of Health and Sciences, Istanbul, Türkiye*

[6] *Department of Physics, Faculty of Science, Gebze Technical University, Kocaeli, Türkiye*

[7] *Department of Medical Biochemistry, Faculty of Medicine, Istanbul Atlas University, Istanbul, Türkiye*

** Corresponding author: E-mail: asiye.yurttas@atlas.edu.tr*

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Abstract

This study evaluates the photodynamic inactivation potential of a coumarin-functionalized zinc(II) phthalocyanine derivative against methicillin-resistant *Staphylococcus aureus* (MRSA) ATCC 33591. The antimicrobial and cytotoxic effects of the derivative were assessed under dark and laser-irradiated (658 nm) conditions. Minimum inhibitory concentration (MIC) values were determined, and the photodynamic response was further explored in MRSA-infected HT-29 (human colorectal adenocarcinoma) and HUVEC (human umbilical vein endothelial) cell models. While the derivative exhibited dose-dependent antibacterial activity, the light-induced reduction in colony-forming units (CFU) was moderate, remaining below a tenfold (1-log10) under the tested conditions. In cell-based assays, the compound showed detectable dark cytotoxicity, which was further enhanced upon laser irradiation. The photodynamic cytotoxic response was more pronounced in infected HT-29 cells than in infected HUVEC cells, with flow-cytometric profiles consistent with predominantly necrotic/late apoptotic cell-death patterns. These findings indicate that the compound exhibits light-enhanced effects in complex infection models; however, further optimization of formulation, photophysical properties, and light dosimetry is required to improve antimicrobial efficacy and selectivity.

Keywords: Methicillin-resistant *Staphylococcus aureus*; microbial pathogens; dose-dependent antibacterial activity; Photodynamic inactivation; Photosensitizing phthalocyanine; Laser

1. Introduction

Staphylococcus aureus is a major human pathogen responsible for a wide spectrum of diseases, ranging from minor skin and soft tissue infections to severe, life-threatening conditions such as bacteremia, pneumonia, and endocarditis.[1] The emergence and global spread of methicillin-resistant *S. aureus* (MRSA) have significantly complicated treatment options, particularly in healthcare settings, due to its resistance to β-lactam antibiotics and other antimicrobial classes.[2] MRSA infections are associated with increased morbidity, mortality, and healthcare costs, highlighting the urgent need for novel diagnostic and therapeutic strategies.[2] *S. aureus* ATCC 33591 is a widely used MRSA reference strain characterized by the presence of the mecA gene, which encodes the penicillin-binding protein PBP2a, conferring resistance to methicillin and relat-

ed antibiotics.[3] This strain serves as a valuable model in microbiological research for evaluating antimicrobial susceptibility, resistance mechanisms, and biofilm-forming capabilities.[4,5] The increasing prevalence of multidrug-resistant bacteria such as MRSA has driven the search for alternative antimicrobial approaches. Among these, antimicrobial photodynamic inactivation (aPDI), also known as photodynamic therapy (PDT), has emerged as a promising strategy.[6] aPDI involves the use of a photosensitizer, light of a specific wavelength, and molecular oxygen to generate reactive oxygen species (ROS) that can effectively kill bacteria through oxidative damage to cellular components.[7] Unlike conventional antibiotics, aPDI targets multiple cellular structures and pathways, reducing the likelihood of resistance development.[8]

Phthalocyanines are a class of synthetic photosensitizers that have attracted considerable attention due to their strong absorption in the red/near-infrared region, high singlet oxygen quantum yield, and chemical stability.[9,10] Several studies have demonstrated antibacterial activity of phthalocyanine derivatives against both planktonic and biofilm forms of *S. aureus* when activated by light.[11–13] For example, Dimaano et al.[11] showed that silicon phthalocyanine (Pc 4) exhibited photodynamic antibacterial effects against *S. aureus* only upon light activation. Similarly, other studies have reported dose-dependent and light-dependent inactivation of *S. aureus* using various phthalocyanine-based photosensitizers.[12] In addition, nanoemulsion-encapsulated zinc phthalocyanine has been reported to inactivate *S. aureus* under optimized conditions.[13] Despite these promising results, some studies have raised concerns about the potential for bacteria to develop tolerance to repeated sublethal photodynamic treatments.[14] Therefore, further research is needed to optimize treatment parameters and evaluate the efficacy and safety of phthalocyanine-based photodynamic therapy against clinically relevant MRSA strains.

In this study, we investigated the antibacterial effect of a coumarin-functionalized zinc(II) phthalocyanine derivative against *S. aureus* ATCC 33591 under both dark and laser-irradiated conditions and assessed its response in MRSA-infected HT-29 and HUVEC cell models.

2. Experimental

2.1. Photosensitizer

The photosensitizer used in this study was a tetra-7-oxy-3-biphenylcoumarin-substituted zinc(II) phthalocyanine derivative, previously synthesized and characterized by Gök et al.[15] The compound exhibits a maximum absorption (λ_{max}) at 690 nm (Figure 1). Considering the known tendency of phthalocyanines to aggregate in aqueous media, all experiments were performed in PBS without additional solubilizing agents; this limitation is considered in the interpretation of the results.

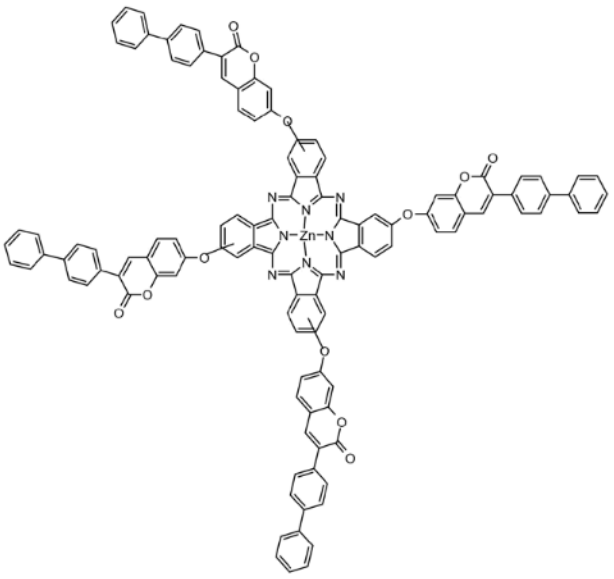


Figure 1. Chemical structure of tetra-7-oxy-3-biphenylcoumarin substituted zinc(II) phthalocyanines.

2.2. Light source and dosing

Photodynamic inactivation experiments were performed using a custom-built laser system with a central wavelength of 658 nm and a Full Width at Half Maximum (FWHM) below 1 nm. The laser delivered an output power of 17.7 mW with a circular beam diameter of 6 mm, corresponding to an irradiance of 62.5 mW/cm^2. Samples were irradiated for 60 s, resulting in a fluence of 3.75 J/cm^2 per well. The system was calibrated before each experiment using a power meter. Light transmission through microplate lids was accounted for when determining the effective fluence. Because the beam diameter is smaller than the well diameter of a standard 96-well plate, spatial variations in irradiance across the well may occur; this limitation is acknowledged.

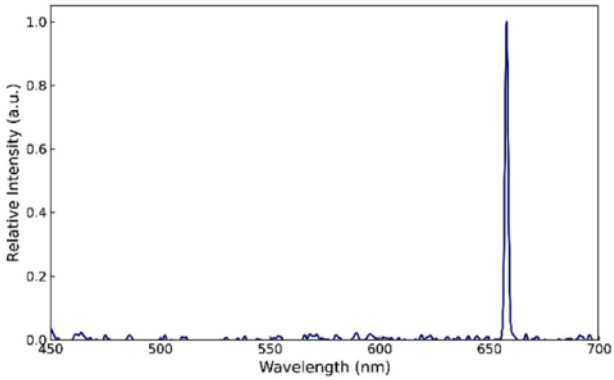


Figure 2. Emission spectrum of the laser.

2.3. Bacterial strain and culture conditions

Staphylococcus aureus ATCC 33591 (MRSA) was obtained as KWIK-STIK™ (Microbiologics, Minnesota, USA).

Bacteria were cultured aerobically in Brain Heart Infusion (BHI) broth and on Tryptic Soy Agar (TSA; BD Difco, Germany) at 37 °C for 18–24 h. The inoculum was adjusted to 0.5 McFarland (~1.5×10^8 CFU/mL) using a densitometer. For assays, cultures were diluted in Mueller–Hinton Broth (MHB; Merck, Germany) and subsequently in PBS to obtain a working inoculum of ~1.13×10^7 CFU/mL.

2.4. Antibacterial activity assay

The dark incubation time (2 h) was selected based on commonly used aPDT pre-incubation conditions reported for phthalocyanine-type photosensitizers, which aim to allow sufficient adsorption/association with bacterial cells and to approach a quasi-equilibrium distribution prior to irradiation.

Phthalocyanine solutions were prepared at 10, 5, 1, and 0.5 µmol/L in PBS. For each concentration, 50 µL of the photosensitizer solution was dispensed into 96-well plates (in duplicate), followed by the addition of 50 µL of bacterial suspension (final volume: 100 µL per well). After incubation at 37 °C for 2 h, one plate was irradiated (LIGHT) for 60 s (3.75 J/cm^2), while the parallel plate was kept in the dark (DARK). Serial dilutions (10^{-3} and 10^{-4}) were prepared from each well, and 10 µL aliquots were plated onto TSA. Plates were incubated at 37 °C for 18–24 h, and CFU were counted. CFU values were calculated considering dilution factors and plating volumes. Qualitative metabolic activity was assessed by the resazurin color change (Figure 3).

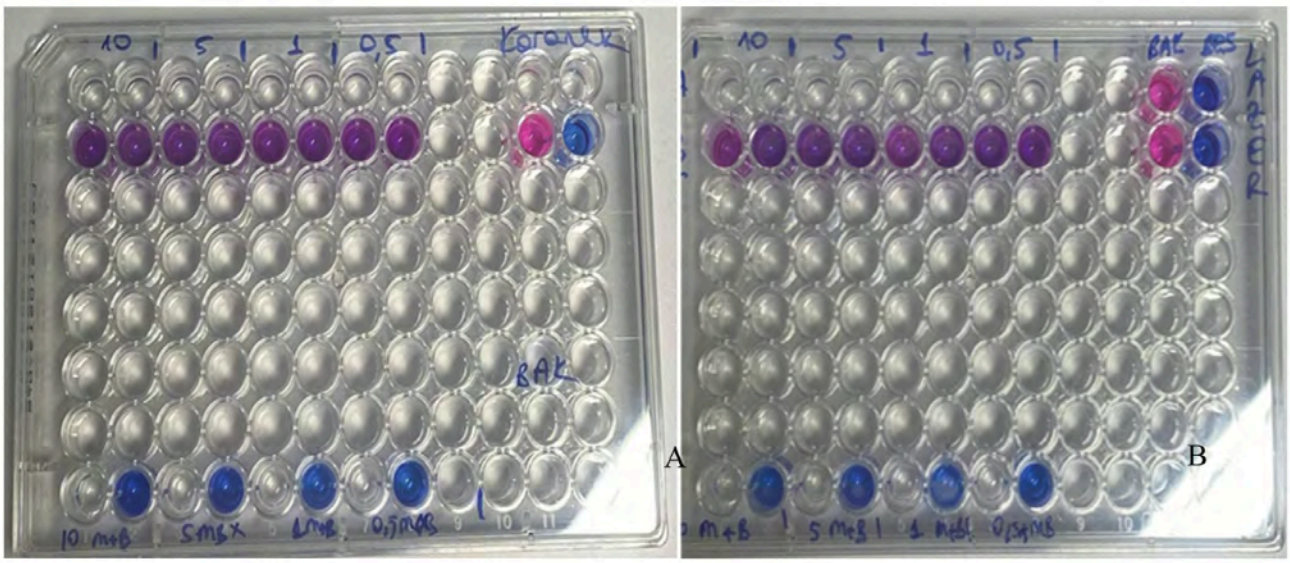


Figure 3: Resazurin-dyed microplate under (a) dark and (b) light conditions.

2.5. Establishment of host cell infection

HT-29 and HUVEC cells were cultured in DMEM/F12 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin at 37 °C in a humidified 5% CO_2 atmosphere. Cells were seeded at 1×10^4 cells per well in 96-well plates and allowed to adhere for 24 h. *Staphylococcus aureus* ATCC 33591 was grown overnight in BHI at 37 °C, diluted 1:50, and incubated for 1.5 h to reach ~1×10^8 CFU/mL, then diluted in serum-free DMEM/F12 to ~1×10^6 CFU/mL. After removal of culture medium and one PBS wash, 100 µL of bacterial suspension was added to each well and incubated for 1 h at 37 °C. Non-adherent bacteria were removed by PBS washes. To eliminate extracellular bacteria, cells were incubated for 2 h at 37 °C in antibiotic-free medium containing either 10 µg/mL lysostaphin or 100 µg/mL gentamicin, washed, and replenished with fresh medium. Sterility of culture supernatants was confirmed by agar plating 24 h post-infection.[16] Intracellular infection levels were not directly quantified and are considered a limitation.

2.6. Photodynamic treatment of infected cells

The infection/incubation period (4 h) was selected in accordance with widely used *in vitro S. aureus* infection models, where a 2–4 h window is commonly applied to permit bacterial adhesion and internalization and to establish a reproducible infection state prior to downstream treatments.

Following infection, cells were washed and treated with phthalocyanine at 0.25–20 µmol/L (prepared from a 54.7 µmol/L stock solution) for 4 h at 37 °C. After incubation, cells were washed with PBS and fresh medium was added. Light-designated wells were irradiated for 60 s (658 nm; 3.75 J/cm^2), whereas dark controls received no light exposure. Cell metabolic activity was measured by the MTT assay (0.4 mg/mL MTT, 3 h at 37 °C), followed by dissolution of formazan crystals in DMSO and absorbance measurement at 540 nm.

2.7. Apoptosis/necrosis analysis by flow cytometry

After infection, cells were treated with 5 μmol/L phthalocyanine for 4 h at 37 °C, washed, and incubated in fresh medium. Light groups were irradiated for 60 s (658 nm; 3.75 J/cm^2), while dark controls were not irradiated. Cells were harvested, washed, and stained with Annexin V–FITC and 7-AAD according to the manufacturer's instructions. Samples were incubated for 15 min at room temperature (22–25 °C) in the dark and analyzed by flow cytometry. Because gating strategy and staining can influence quantitative outputs, results are interpreted cautiously.

Table 1. Experimental groups

Experimental groups for HT-29 cell:		**Experimental groups for HUVEC cell:**	
1. Cells only		1. Cells only	
2. Infected cells		2. Infected cells	
3. Infected cells + 5 μM phthalocyanine (DARK)		3. Infected cells + 5 μM phthalocyanine (DARK)	
4. Infected cells + 5 μM phthalocyanine (LIGHT)		4. Infected cells + 5 μM phthalocyanine (LIGHT)	
HT-29 cell:	**HUVEC cell:**		
Group No.	**Description**	**Group No.**	**Description**
1	Cells only (unstained)	**1**	Cells only (unstained)
2	Cells only (stained)	**2**	Cells only (stained)
3	Infected cells (unstained)	**3**	Infected cells (unstained)
4	Infected cells (stained)	**4**	Infected cells (stained)
5	Infected cells + drug (dark)	**5**	Infected cells + drug (dark)
6	Infected cells + drug (light)	**6**	Infected cells + drug (light)

3. Results and discussion

3.1. Antibacterial activity and CFU analysis

The antibacterial activity of the phthalocyanine derivative against *S. aureus* (MRSA ATCC 33591) was evaluated across 0.5–10 μmol/L under dark conditions and after laser irradiation (658 nm; 3.75 J/cm^2) (Figure 4, Table 2). Under dark conditions, bacterial counts remained in the 10^4–10^5 CFU/mL range, indicating limited antibacterial activity in the absence of light. Upon irradiation, a measurable reduction in CFU was observed at higher concentrations (5 and 10 μmol/L). However, the magnitude of reduction remained below 1 $\log_{10}$ under the tested conditions, suggesting moderate aPDI activity rather than robust inactivation. This limited efficacy may be influenced by the aggregation tendency of phthalocyanines in aqueous buffers, which can suppress photochemical ROS generation.

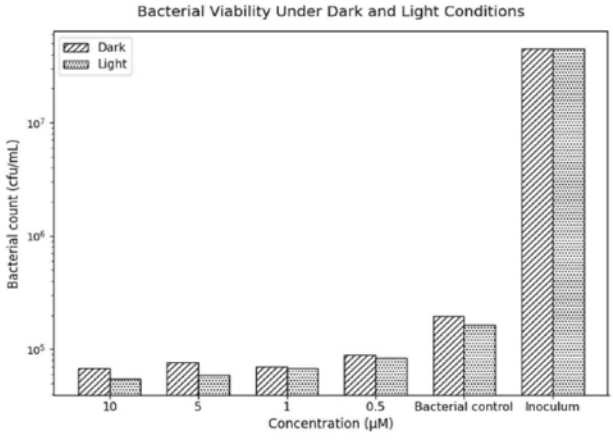


Figure 4: Bacterial cell viability under dark and light conditions at different concentrations of the compound.

Table 2. Viable bacterial count (CFU/mL)

Concentration (μmol/L)	Dark (CFU/mL)	Light (CFU/mL)
10	6.8×10^4	5.5×10^4
5	7.67×10^4	5.9×10^4
1	7.0×10^4	6.8×10^4
0.5	8.85×10^4	8.43×10^4
Bacterial control	1.96×10^5	1.64×10^5
Inoculum	4.5×10^7	4.5×10^7

3.2. Phototherapeutic activity on infected cell models

The response of MRSA-infected HUVEC and HT-29 cells to the phthalocyanine derivative was evaluated by the MTT assay after 4 h exposure (Figures 5 and 6). In infected HUVEC cells, the compound caused concentration-dependent reductions in MTT signal under both dark and irradiated conditions, indicating detectable intrinsic (dark) cytotoxicity and only a modest additional light effect under the tested irradiation parameters. In infected HT-29 cells, reductions in MTT signal were more pronounced, and irradiation further decreased metabolic activity compared with dark controls. Because MTT primar-

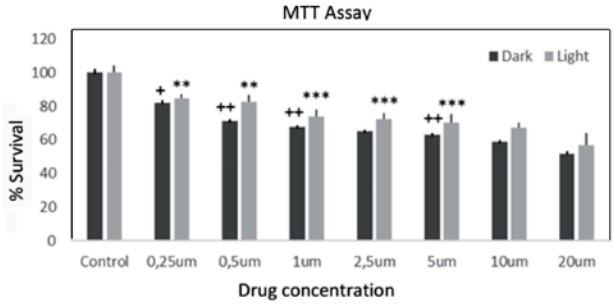


Figure 5: The viability (%) of infected HUVEC cells based on the MTT assay results. +; $p < 0.05$, ++; $p < 0.01$, *; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$.

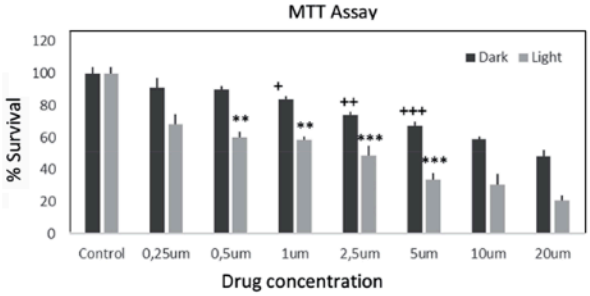


Figure 6: The viability (%) of infected HT-29 cells based on the MTT assay results.

ily reflects metabolic activity, these results are interpreted as changes in cellular metabolic state rather than direct proof of selective photodynamic killing. Nonetheless, the differential response between the two cell types suggests that host-cell context (e.g., uptake, redox buffering, and infection-related stress) may modulate the observable PDT response.

3.3. Cell death pathways by annexin V/7-AAD

Annexin V/7-AAD staining was performed to examine cell-death patterns following treatment with 5 μmol/L phthalocyanine, with or without irradiation (Figure 7). In both infected HUVEC and infected HT-29 cultures, the dominant pattern was consistent with loss of membrane integrity (necrotic/late-stage populations). Differences between dark and light groups were limited under the tested conditions, suggesting that (i) intrinsic compound toxicity and infection-induced stress may dominate at this dose, and/or (ii) the applied light dose was insufficient to produce a strongly separated light-triggered profile. These results should be considered preliminary and warrant further experiments with optimized dosimetry and independent validation of infection burden.

3.4. Mechanistic considerations and limitations

Phthalocyanine photosensitizers typically act through photoactivation and subsequent ROS generation. In the present study, ROS production and aggregation state were not directly measured; therefore, mechanistic

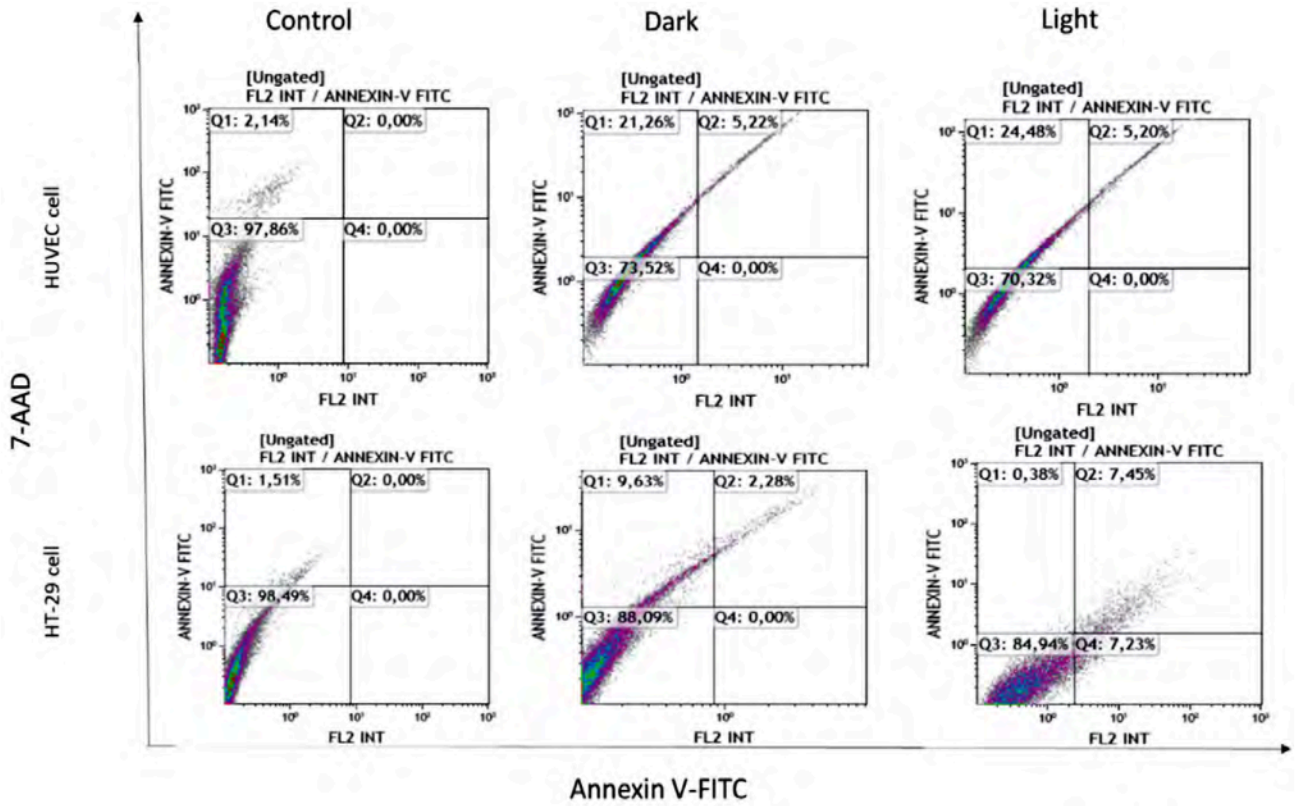


Figure 7: Analysis of Annexin-V/7AAD staining in infected cells

interpretations are limited. Given the hydrophobic character of the compound and the propensity of phthalocyanines to aggregate in aqueous media, aggregation may reduce effective cellular uptake and photochemical efficiency, contributing to the moderate antibacterial performance. Differences observed between HT-29 and HUVEC models may also reflect cell-type-dependent uptake and redox buffering, as well as differences in bacterial localization (intracellular vs extracellular), although intracellular infection levels were not quantified in this study.

In general, photodynamic antimicrobial outcomes are highly dependent on photosensitizer class, experimental medium, and applied light dosimetry, and similar light-dependent effects have been reported across related systems.[17,20,21] Cell-type-specific antioxidant capacity and redox buffering can further modulate susceptibility to ROS-mediated injury in mammalian infection models.[22] Moreover, *S. aureus* can invade non-phagocytic host cells and elicit distinct host responses, which may influence the effective proximity between bacteria, host cells, and the photosensitizer; however, bacterial localization was not directly assessed in the present work.[18,23] *S. aureus* also expresses carotenoid antioxidants such as staphyloxanthin that can mitigate oxidative stress and potentially reduce PDT susceptibility.[19] Depending on ROS dose and cellular context, oxidative injury may manifest predominantly as necrotic/late-stage death rather than classical apoptosis. [24]

5. Conclusion

This study demonstrates that the coumarin-functionalized zinc(II) phthalocyanine derivative exhibits light-enhanced effects in MRSA-related *in vitro* models; however, under the tested conditions, antibacterial inactivation remained moderate (below 1 $\log_{10}$ CFU reduction). In infected cell models, the compound produced concentration-dependent decreases in metabolic activity under both dark and irradiated conditions, with a more pronounced light-associated response in infected HT-29 cells than in infected HUVEC cells. Overall, the findings suggest that photodynamic performance is strongly influenced by concentration, biological environment, and likely the aggregation/uptake behavior of the phthalocyanine. Further work incorporating photophysical/photochemical characterization (e.g., aggregation and ROS assays), improved formulation or delivery strategies, and optimized light dosimetry will be necessary to enhance antimicrobial efficacy and to better define selectivity in complex infection models.

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Conflict of interest statement

The authors have no conflicts of interest to declare.

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Availability of data and materials

Authors can confirm that all relevant data are included in the article and/or its supplementary information files.

Authors contributions

MB: Conceptualization; Data curation; Formal analysis; Investigation; Methodology; Validation; Visualization; Writing-original draft.
TE: Conceptualization; Formal analysis; Funding acquisition; Investigation; Methodology; Project administration; Resources; Supervision; Validation; Writing-review & editing.
ÇA: Investigation; Validation; Writing-original draft.
OOK: Investigation; Validation; Writing-original draft.
KÇ: Formal analysis; Investigation; Visualization; Writing-original draft.
AGY: Funding acquisition; Resources; Supervision; Writing-review & editing.

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Povzetek

Ta študija ocenjuje potencial fotodinamične inaktivacije derivata cink(II) ftalocianina, funkcionaliziranega s kumarinom, proti meticilin-rezistentnemu *Staphylococcus aureus* (MRSA) ATCC 33591. Protimikrobni in citotoksični učinki derivata so bili ocenjeni v temi in v pogojih obsevanja z laserjem (658 nm). Določene so bile vrednosti minimalne inhibitorne koncentracije (MIC), fotodinamični odziv pa je bil dodatno raziskan na celičnih modelih HT-29 (človeški kolorektalni adenokarcinom) in HUVEC (endotelij človeške popkovne vene), okuženih z MRSA. Medtem ko je derivat pokazal koncentracijsko odvisno protibakterijsko aktivnost, je bilo svetlobno povzročeno zmanjšanje števila kolonijskih enot (CFU) zmerno in je v preizkušenih pogojih ostalo pod zmanjšanjem za 1 $\log_{10}$. V celičnih testih je spojina pokazala zaznavno citotoksičnost v temi, ki se je ob laserskem obsevanju še okrepila. Fotodinamični citotoksični odziv je bil izrazitejši v okuženih celicah HT-29 kot v okuženih celicah HUVEC, pri čemer so bili profili, pridobljeni s pretočno citometrijo, skladni s pretežno nekrotičnimi/pozno apoptotičnimi vzorci celične smrti. Ti rezultati kažejo, da spojina v kompleksnih modelih okužbe kaže učinke, ki jih okrepi svetloba; vendar je za izboljšanje protimikrobne učinkovitosti in selektivnosti potrebna nadaljnja optimizacija formulacije, fotofizičnih lastnosti in dozimetrije svetlobe.

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