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Meso-substituted cationic 3- and 4-N-Pyridylporphyrins and their Zn(II) derivatives for antibacterial photodynamic therapy

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Photodynamic inactivation of microorganisms known as antibacterial photodynamic therapy (APDT) is one of the most promising and innovative approaches for the destruction of pathogenic microorganisms. Among the photosensitizers (PSs), compounds based on cationic porphyrins/metalloporphyrins are most successfully used to inactivate microorganisms. Series of

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meso-substituted cationic pyridylporphyrins and metalloporphyrins with various peripheral groups in the third and fourth positions of the pyrrole ring have been synthesized in Armenia. The aim of this work was to determine and test the most effective cationic porphyrins and metalloporphyrins with high photoactivity against Gram negative and Gram positive microorganisms. It was shown that the synthesized cationic pyridylporphyrins/metalloporphyrins exhibit a high degree of phototoxicity towards both types of bacteria, including the methicillin-resistant *S. aureus* strain. Zinc complexes of porphyrins are more phototoxic than metal-free porphyrin analogs. The effectiveness of these Zn–metalloporphyrins on bacteria is consistent with the level of singlet oxygen generation. It was found that the high antibacterial activity of the studied cationic porphyrins/metalloporphyrins depends on four factors: the presence in the porphyrin macrocycle of a positive charge (+4), a central metal atom (Zn^{2+}) and hydrophobic peripheral functional groups as well as high values of quantum yields of singlet oxygen. The results indicate that meso-substituted cationic pyridylporphyrins/metalloporphyrins can find wider application in photoinactivation of bacteria than anionic or neutral PSs usually used in APDT.

Keywords: Antibacterial photodynamic therapy; cationic porphyrins/metalloporphyrins; phototoxicity; Zn–metalloporphyrins; singlet oxygen quantum yield; Gram negative and Gram positive bacteria; *S. aureus*; MRSA; *E. coli*; *Salmonella typhimurium*.

1. Introduction

The widespread resistance of microorganisms to antimicrobial drugs has long been recognized as a global problem.[1,2] In connection with the tendency of an increase in the number of such microorganisms, the search and development of alternative approaches to their damage/destruction is extremely important. Photodynamic inactivation of microorganisms known as antibacterial photodynamic therapy (APDT) by means of photosensitive dyes — photosensitizers (PSs) — is one of the most promising and innovative approaches for the destruction of pathogens.[3–5] Currently, work in the field of APDT has acquired a pronounced practical orientation, which is due to the growth of resistance of pathogenic microorganisms to traditional chemotherapy (including antibiotics) and the need to develop alternative methods of their inactivation. There are many studies indicating a wide variety of bacteria (*Staphylococcus aureus*, *Streptococcus pyigenes*, *Clostridium perfingens*, *Escherichia coli*, *Micoplasma hominis*, etc.) and fungi sensitive to APDT.[5–7] The independence of lethal photosensitization from species specificity is a great advantage, since this method in a mixed infection can destroy not only all microorganisms, but also viruses.[8]

The APDT, like the photodynamic therapy (PDT) of tumors, combines three components: the photosensitizer, oxygen and light,[3] which lead to the formation of reactive oxygen species (ROS) (singlet oxygen and free radicals). Reacting with most macromolecules and cellular components (membranes, proteins, DNA, RNA, lipids, sugars, etc.), ROS cause oxidative processes and damage leading to cell death.[9–11] Microorganisms cannot resist this type of impact due to the multiplicity and variety of targets.[12–14] In this case, not only microorganisms are damaged and destroyed, but also yeast, viruses and protozoa.[3,15–20]

Among the photosensitizers for inactivation of microorganisms, compounds based on porphyrins[21] as well as phthalocyanines[22] and phenothiazines[23] have been used with the greatest success, and somewhat less often on the basis of porphycenes.[24] One of the most important factors in the destruction of microorganisms is the charge of the photosensitizer. The effectiveness of neutral PSs (porphyrins and phthalocyanines) is clearly shown against Gram positive bacteria, but they are unable to inactivate Gram negative bacteria, due to the more complex structure of the bacterial wall.[25–27] Phospholipids, complexes of lipoproteins and polysaccharides present in the outer membrane of Gram negative bacteria (*E. coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Haemophilus influenzae*), also inhibit the binding of anionic PS molecules and make them ineffective.[21]

In the development and improvement of the APDT approach for microorganisms, cationic porphyrins have become widespread. Cationic porphyrins greatly facilitate the processing and interpretation of experimental data due to the fact that, in contrast to anionic porphyrins, they do not form aggregates in solution and remain in monomeric form in a wide range of concentrations.[28] Strongly negative components of the cell walls of Gram positive [lipoteichoic acid (LTA)] and Gram negative [lipopolysaccharide (LPS)] bacteria due to electrostatic interactions with cationic groups of PSs provide high affinity for both types of bacteria and, accordingly, high phototoxicity of cationic porphyrins.[14,29–31] The possibilities of synthesis and a variety of cationic porphyrins make it possible to successfully use meso-substituted cationic porphyrins with different charge distributions (tetra-, tri-, di- or mono-cationic) in the inactivation of a wide spectrum of both Gram positive and Gram negative bacteria.[27,32–36] In the last two decades, some meso-substituted cationic pyridylporphyrins and metalloporphyrins with various peripheral groups in the third and fourth positions of the pyrrole ring have been synthesized in Armenia.[37,38] See Fig. 1.

Groups of Dr. L. T. Benov (Kuwait) and Dr. I. Batinic-Haberle (USA) as well as other authors, in the recently published works,[39–42] have studied in detail similar cationic porphyrins like the ones presented in this work in application to APDT.

Among the ROS generated by photosensitizers upon their activation by light, singlet oxygen ($^1O_2$) is the main toxic molecule.[43] The amount of singlet oxygen generated during PDT/APDT is an important indicator of PS efficiency. The luminescence intensity of $^1O_2$ is proportional to the number of formed singlet oxygen molecules.[44,45] Study and determination of the quantum yield of singlet oxygen (Φ_Δ) of cationic porphyrins is an important stage in the study of inactivation of microorganisms.[14]

The aim of this work is the determination and testing of the most effective meso-substituted cationic pyridylporphyrins and metalloporphyrins, which have high photoactivity against Gram positive and Gram negative microorganisms.

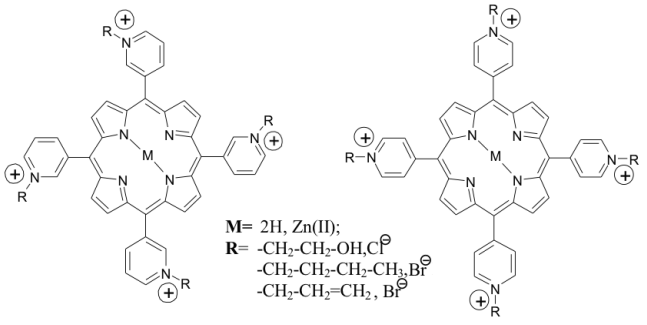


Fig. 1. Water-soluble meso-substituted cationic *N*-quaternary pyridylporphyrins and metalloporphyrins with various peripheral functional groups in the third and fourth positions of the pyrrole ring. *M*: metal and *R*: peripheral functional groups with the counterions Cl^- and Br^-(hydroxyethyl, butyl and allyl).

2. Materials and Methods

2.1. *Cationic porphyrins and metalloporphyrins*

Series of water-soluble meso-substituted cationic *N*-quaternary 3- and 4-pyridylporphyrins and their Zn(II) derivatives with various peripheral functional groups (hydroxyethyl, butyl and allyl) were synthesized in accordance with our previously described methods.[37,38]

The structure of the synthesized compounds was confirmed by infrared and absorption spectroscopies as well as by nuclear magnetic resonance.

The following compounds were used in this work (Fig. 1): meso-tetrakis(*N*-(2′-hydroxyethyl)pyridinium-4(or 3)-il)porphyrin chloride (H_2TOE4PyP and H_2TOE3PyP), meso-tetrakis(*N*-*n*-butylpyridinium-4-il)porphyrin bromide (H_2TBut4PyP), meso-tetrakis(*N*-allylpyridinium-4-il)porphyrin bromide (H_2TAll4PyP) and their corresponding Zn(II) complexes (Zn–TOE4PyP, Zn–TBut4PyP, Zn–TBut3PyP and Zn–TAll4PyP).

The syntheses of 3-*N*-pyridylporphyrins (and the Zn complex) with *n*-butyl and hydroxyethyl side-chains[46,47] were performed employing the same synthetic approach as it has been described for *para* 4-*N*-pyridylporphyrins and their metallocomplexes earlier.[38] Briefly, a solution of H_2T3PyP in dimethyl formamide (DMF) was mixed with a suitable alkylating reagent (*n*-butyl bromide or 2-hydroxyethyl chloride) and heated to reflux. The progress of the reaction was followed by thin-layer chromatography (TLC) as well as by UV–visible spectrophotometry. Metal-free porphyrins (H_2TBut3PyP and H_2TOE3PyP) were precipitated from the reaction

mixture with diethyl ether, filtered and washed with diethyl ether. The resulting porphyrins were dissolved in ethanol, metallated by the addition of $ZnBr_2$ and heated under reflux. The progress of the reaction was monitored as described above. Upon completion of metallization, the Zn complexes (Zn–TBut3PyP and Zn–TOE3PyP) were precipitated and washed with acetone.

All other chemicals were of analytical grade and were purchased from commercial sources (Sigma-Aldrich).

2.2. *Determination of the quantum yield of singlet oxygen generation of photosensitizers (Φ_Δ)*

The quantum yields of the singlet oxygen formation (Φ_Δ) of pyridylporphyrins/metalloporphyrins were determined according to the literature previously.[48] Time-resolved luminescence of singlet oxygen in the near-infrared region (NIR) was measured on a nanosecond laser NIR spectrometer.[49,50] Samples (porphyrin solutions) were excited by laser pulses (10 ns, energy $\leq 1\mu$J) at a repetition rate of 5 kHz at $\lambda = 532$ nm (Nd:YAG laser DTL-314QT, Laserexport Co., Ltd., Russia). Quantum yields of singlet oxygen formation were determined by a relative method using meso-tetra-(*N*-methyl-4-pyridyl)porphyrin tosylate as a standard, $\Phi_\Delta = 0.77$.[51]

2.3. *Microorganisms*

We used the bacterial strains *E. coli* K-12 (from the collection of the Scientific and Production Center "Armbiotechnology" NAS RA), *Salmonella typhimurium* G-38, *Staphylococcus epidermidis* (*S. epidermidis*) and *S. aureus* (from the collection of A. Aleksanyan Research Institute of Epidemiology, Virology and Medical Parasitology, Ministry of Health of RA) and *S. aureus* 209 P and methicillin-resistant *S. aureus* (MRSA) (from the collection of the L. A. Tarasevich State Research Institute for Standardization and Control of Biological Medicines, Russia). The microorganisms were grown aerobically in L-bouillon (LB medium) in an orbital shaker at 32–37°C. The stationary phases of microorganisms after 18–20 h were harvested by centrifugation at 4000 rpm for 10 min, washed twice with phosphate saline buffer (PBS) at a pH of 7.4 and suspended in PBS.

2.4. *Dark- and photo-toxicity of porphyrins for microorganisms*

The toxic or phototoxic effects of porphyrins and metalloporphyrins in the dark or upon light exposure on Gram positive and Gram negative microorganisms were determined by measuring the extent of growth inhibition and reduction of cells survival after treatment with porphyrins according to the literature.[50] Samples with a volume of 1 mL containing 0.9 mL of a suspension of microorganisms in LB medium with a final cell concentration of $10^8 - 2 \times 10^9$ cells/mL as well as a solution of the corresponding porphyrin (0.1 mL) in phosphate buffer (PBS) were prepared and incubated at 32–37°C for 22–24 h under aerobic conditions. Microbial survival was determined with the modified version of Miles *et al.*'s method.[52,53] For determination of photosensitizers' phototoxicity (Secs. 3.2.1–3.2.3), 1 mL of a cells suspension with the selected photosensitizer (concentrations between 0.001 μg/mL and 10 μg/mL) was incubated for 10 min in the dark at room temperature and then illuminated with white light (at an illumination power density of 30 mW/cm^2). During illumination (up to 30 min) the cells were kept at 30–37°C and stirred. The percentage of survival was determined by dividing the number of colony-forming units (CFU) from cells illuminated with a photosensitizer by the number of CFU from cells illuminated without a photosensitizer.

Before the start of experiments with MSSA and MRSA (Sec. 3.2.4), 2 mL of a culture of microorganisms in LB medium was centrifuged at 1500× *g* for 10 min and washed twice with PBS. The cell pellet was resuspended in PBS to obtain an inoculum of approximately 10^8 colony-forming units (CFU/mL). Then, 100 μL of a standardized suspension of the test strains was added to each well of a 96-well polystyrene plate. The wells were divided into four groups: (1) with the addition of 100 μL of PBS — control, (2) with the addition of 100 μL of photosensitizers — control of the toxicity of photosensitizers, (3) with the addition of 100 mL of PBS — to assess the effect of illumination alone — and (4) with the addition of 100 μL of photosensitizers — to assess the effectiveness of the photodynamic effects of illumination and photosensitizers. Then the plate was shaken for 20 min in an orbital shaker without access to light. Well groups 1 and 2 were continued with incubation in

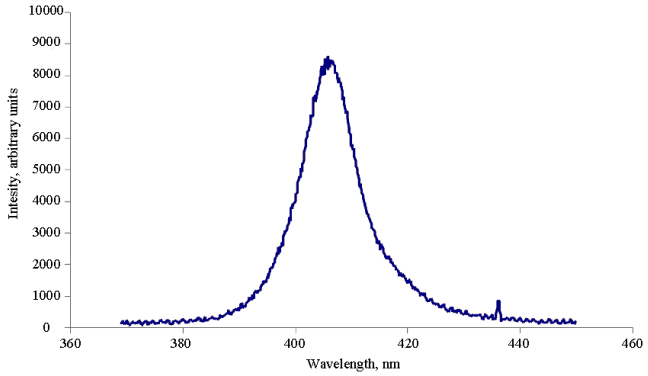


Fig. 2. The 405-LED emission spectrum.

the dark as a general control and to determine the initial concentration of bacteria in suspensions. Well groups 3 and 4 were exposed to illumination [light-emitting diode (LED) with the maximum emission of 405 nm (Fig. 2), 70 mW/cm^2]. After illumination or incubation in the dark, samples were serially diluted and put onto LB agar plates. The plates were then incubated aerobically at 37°C for about 24 h. To assess the photodynamic effect, bacterial cell survival ($\log_{10}$CFU) was calculated in all groups of wells.

2.5. *Statistical analysis*

All experiments were carried out in five biological replicates. Statistical analyses were performed using STATISTICA data analysis software (version 10.0) and MS Excel. The quantitative variables were characterized by the arithmetic mean, standard deviation and 95% confidence interval. The statistical significance of the differences between the two groups was processed using Student's *t*-test. In all calculations, a *p*-value of 0.05 was used as the threshold for statistical significance.

3. Results and Discussion

The photocytotoxicities of 10 water-soluble meso-substituted *N*-quaternary cationic porphyrins (Fig. 1), differing in peripheral groups, the position of groups in the pyrrole ring and the presence/absence of zinc metal in the central position of pyridine macrocycle, were tested. The effectiveness of the compounds against microorganisms was evaluated based on determining the percentage of survival of the number of CFU from cells irradiated with the investigated photosensitizer and cells irradiated without a photosensitizer.

3.1. *Spectral properties and the quantum yield of singlet oxygen formation by cationic porphyrins*

The spectral characteristics as well as the efficiency of photosensitized singlet oxygen formation by cationic porphyrins have been studied in detail earlier.[48,54] Some of the characteristic features should be noted. It was shown that the chemical structure of the pyridyl substituent (hydroxyethyl, butyl and allyl) does not significantly affect the contours and intensity ratios of bands in the absorption and fluorescence spectra of porphyrins. At the same time, the transition from [3-pyridyl] to [4-pyridyl] porphyrins is accompanied by (i) a shift of the band maxima in the absorption spectra to longer wavelengths by 5–7 nm (Fig. 3), (ii) a change in the shape of the fluorescence spectra (Fig. 4) and (iii) a decrease in the fluorescence lifetime by more than 1.5 times. These changes are assumed to be

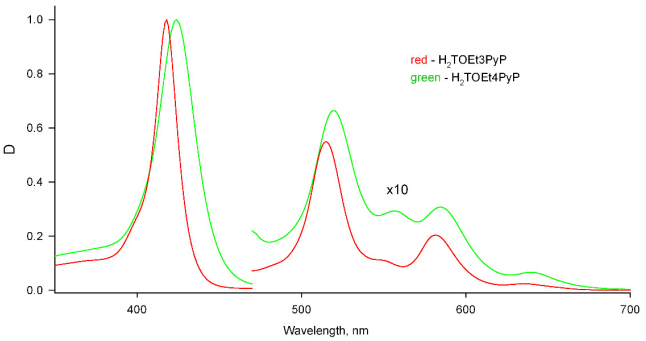


Fig. 3. Absorption spectra of aqueous solutions of cationic porphyrins H_2TOE3PyP and H_2TOE4PyP.

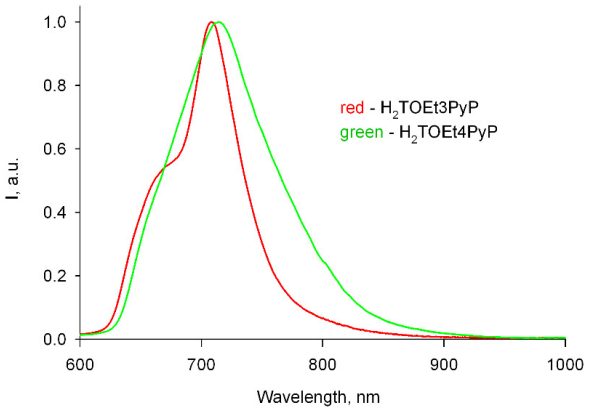


Fig. 4. Normalized fluorescence spectra of aqueous solutions of cationic porphyrins H_2TOE3PyP and H_2TOE4PyP. The excitation wavelength is $\lambda_{ex} = 418$ nm.

associated with the fact that a charge transfer state is located near the S_1-state of the porphyrins, and the degree of mixing of these states is determined by free rotation of the pyridyl rings. From geometric considerations, it is clear that the rotation of pyridyl rings encounters obstacles in [3-pyridyl] porphyrins and can be carried out rather freely in [4-pyridyl] porphyrins.

The incorporation of the Zn ion into the porphyrin macrocycle leads to a change in the symmetry of porphyrin molecules,[55] two Q-absorption bands being formed in the spectral region of 500–700 nm (Fig. 5) instead of four (Fig. 3). The behavior of the spectral characteristics of Zn–porphyrins during the transition from [3-pyridyl] to [4-pyridyl] metalloporphyrins is similar to that for metal-free porphyrins.

The quantum yields of the photosensitized singlet oxygen formation were also determined.[48] It was found that all the studied porphyrins have sufficiently high Φ_Δ values. For the metal-free pyridylporphyrins $\Phi_\Delta = 0.77 \pm 0.04$ and for the metalloporphyrins $\Phi_\Delta = 0.89 \pm 0.04$. It was found that the structure of the substituents and their position on the pyridyl ring do not significantly affect the efficiency of photosensitized singlet oxygen formation. The increase in Φ_Δ for Zn complexes is associated with the heavy atom effect.[55] It is known that the introduction of a metal atom into the porphyrin macrocycle leads to an additional interaction between the singlet and triplet states of the porphyrin and to an increase in the intersystem crossing quantum yield, which is directly related to the quantum yield of the photosensitized singlet oxygen formation.[55,56]

It should be noted that the efficiency of the photosensitized formation of singlet oxygen by the studied porphyrins exceeds the Φ_Δ values for most of the known photosensitizers.[57,58] Therefore, it can be expected that the studied cationic meso-substituted pyridylporphyrins/metalloporphyrins are of high efficiency for microorganism photoinactivation, which is shown below.

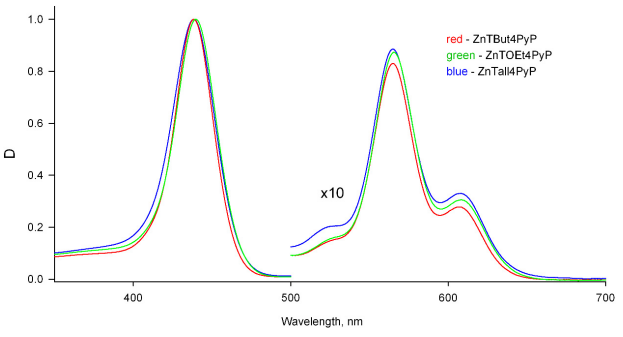


Fig. 5. Absorption spectra of aqueous solutions of cationic metalloporphyrins Zn–TBut4PyP, Zn–TOE4PyP and Zn–TAll4PyP.

Note also that for the ionized aryl substituents, upon radiation inactivation, insignificant differences are observed in the quantum yields of fluorescence and phosphorescence for the similar porphyrins but with different counterions, which are associated with changes in the electronic energy levels in the porphyrin–counterion system.[59]

The shortcoming of display of experimental data for concentrations of porphyrins presented in μg/mL instead of mol/L can impact on the value of generation of singlet oxygen, especially when comparing H_2TOE4PyP (molecular weight of 941 g/mol) with Zn–TBu4PyP (molecular weight of 1230 g/mol). When singlet oxygen is generated by porphyrins with the same weight, expressed in μg/mL, the number of centers generating singlet oxygen for Zn–TBu4PyP will be less than that for H_2TOE4PyP due to the higher molecular weight of Zn–TBu4PyP. It is more correct to carry out these measurements at the same molar concentrations (in mol/L), when the number of centers generating singlet oxygen is the same for all studied compounds. Such measurements of the quantum yield of singlet oxygen generation (Φ_Δ) with the same concentrations (expressed in mol/L) for a number of cationic pyridylporphyrins showed significantly high Φ_Δ for Zn-containing metalloporphyrins, especially for Zn–TBu4PyP.[48] In general, the picture of the activity (Figs. 6–8) of the studied cationic pyridylporphyrins/metalloporphyrins in terms of the presentation of their concentrations in one form or another (μg/mL or mol/L) will not change, and from a practical point of view it is more convenient to use the concentrations expressed in μg/mL.

3.2. *Photoinactivation of microorganisms*

In connection with the possibility of directional synthesis of porphyrins/metalloporphyrins containing various functional groups differing in the position of groups in the pyrrole ring, the task was set to influence the APDT of Gram positive and Gram negative bacteria *in vitro*. The efficiency of photoinactivation (phototoxicity) of bacteria with

new cationic porphyrins/metalloporphyrins was studied for the bacteria that are known[14] to be involved in nosocomial infections [*S. aureus*/Gram (+), *S. epidermidis*/Gram (+), *E. coli*/Gram (−) and *Salmonella typhimurium*/Gram (−)].

3.2.1. *Dark toxicity study of cationic pyridylporphyrins/metalloporphyrins*

One of the main requirements for a PS is its zero or extremely low dark and high light toxicities (phototoxicities) at therapeutic doses.[60–62] Therefore, the determination of the concentration of porphyrins, which is not toxic to microorganisms under dark conditions, is necessary for photodynamic studies. In this regard, at the first stage of the research, a preliminary selection was carried out from the studied six cationic porphyrins/metalloporphyrins on the basis of the highest antibacterial activity in dark conditions*in vitro*. In the studied porphyrins, nontoxic concentrations (the concentration that does not cause the death of microorganisms) and the minimum bactericidal concentrations (MBCs — the concentrations that cause 100% death of microorganisms) were determined.

Preliminary selection of these porphyrins was carried out on the microorganism *E. coli* K-12 under *in-vitro* conditions. This microorganism is a convenient model for studying the effectiveness of porphyrins in connection with the manifestation of significant resistance of *E. coli* to many neutral and anionic PSs.[21,63,64] The results of experiments to determine the bactericidal activity of six studied cationic pyridylporphyrins/metalloporphyrins on *E. coli* K-12 in dark conditions are presented in Table 1.

Comparison of antibacterial activity (dark toxicity) showed a higher efficiency of action of Zn–metalloporphyrins with butyl or allyl functional groups (lower values). It is likely that the presence of these hydrophobic groups in the porphyrin molecule leads to a decrease in MBC compared to porphyrins with an oxyethyl group, which contains an OH hydrophilic end. Apparently, the binding of porphyrins to cells is regulated by the degree of hydrophobicity of peripheral functional groups, which promotes a stronger association with the outer membrane of *E. coli* cells and an increase in the penetration of the studied pyridylporphyrins.

The final results of studies of bactericidal activity (nontoxic concentrations and minimum bactericidal concentrations) of six cationic pyridylporphyrins/metalloporphyrins in relation to *E. coli* K-12 in dark conditions are presented in Table 2.

Data of Table 2 indicate a higher degree of dark activity of cationic pyridylporphyrins, for which the MBCs and nontoxic concentrations are somewhat higher than those of Zn–metalloporphyrins. The results obtained were used for further experiments in order to reveal the photodynamic activity (phototoxicity) of the studied cationic pyridylporphyrins/metalloporphyrins.

3.2.2. *Study of the photodynamic inactivation of the E. coli K-12 bacterium by cationic pyridylporphyrins/metalloporphyrins*

As noted above, studies of the light activity (phototoxicity) of cationic pyridylporphyrins/metalloporphyrins should be carried out at nontoxic concentrations. In this regard, the study of the

Table 1. Survival (%) of cells of the microorganism *E. coli* K-12 after the action of series of cationic pyridylporphyrins/metalloporphyrins in dark conditions.

		Porphyrin concentrations in suspension (μg/mL)				
No.	Porphyrins	0 (Control)	10	20	50	100
1	H_2TOE4PyP	100 ± 2.3	98.2 ± 4.8	97.4 ± 9.3	75.1 ± 7.4	39.3 ± 5.9
2	Zn–TOE4PyP	98.1 ± 5	98.5 ± 3.1	75.1 ± 4.1	57.4 ± 5.5	12.1 ± 4.3
3	H_2TBut4PyP	98.4 ± 3.9	95.4 ± 9.2	76.4 ± 5.9	39.4 ± 7.3	0
4	Zn–TBut4PyP	97.3 ± 6.7	58.2 ± 5.3	23.3 ± 8.1	9 ± 2.7	0
5	H_2TAll4PyP	98.2 ± 4.8	98.4 ± 4.3	79.2 ± 2.7	46 ± 8.3	2.1 ± 0.8
6	Zn–TAll4PyP	99.1 ± 5	91 ± 2.2	21.4 ± 7.2	9.3 ± 5.1	0

Notes: Initial cell titer: 1×10^9 CFU/mL; control: samples without the addition of porphyrins; $p < 0.05$; and $n = 5$.

Table 2. Bactericidal activity of cationic pyridylporphyrins/metalloporphyrins in dark conditions in relation to the microorganism *E. coli* K-12.

No.	Porphyrins	Nontoxic concentration (μg/mL)	MBC (μg/mL)
1	H_2TOE4PyP	20 ± 2.6	320 ± 10.5
2.	H_2TAll4PyP	10 ± 1.1	105 ± 4.3
3.	H_2TBut4PyP	7 ± 0.7	100 ± 3.1
4.	Zn–TOE4PyP	10 ± 1.4	200 ± 5.7
5.	Zn–TAll4PyP	7 ± 0.5	75 ± 2.4
6.	Zn–TBut4PyP	5 ± 0.2	75 ± 2

Note: Initial cell titer: 1×10^9 CFU/mL; control: microorganisms without the addition of porphyrins; $p < 0.05$; and $n = 5$.

phototoxicity of synthetic porphyrin derivatives was carried out at concentrations not exceeding 5–10 μg/mL. The study of the phototoxicity of cationic pyridylporphyrins/metalloporphyrins on the Gram negative bacterium *E. coli* K-12 was carried out with the following cationic pyridylporphyrins: TOE4PyP, TBut4PyP, TAll4PyP and their zinc complexes. Illumination was carried out using a white light source at a power density of 30 mW/cm^2 for 5–30 min.

Table 3 shows the data of photoinactivation of *E. coli* K-12 cells depending on the concentration of cationic pyridylporphyrins/metalloporphyrins (0.01–10 μg/mL) under irradiation for 30 min. As can be seen from the presented data, the MBC of the studied pyridylporphyrins/metalloporphyrins varies from 2 μg/mL to 10 μg/mL. The metalloporphyrins Zn–TAll4PyP and Zn–TBut4PyP were found to be the most effective, which at a concentration of 2 μg/mL lead to 100% death of *E. coli* K-12 microorganisms.

The results of studies of the photodynamic activity of six proposed cationic pyridylporphyrins in comparison with the known PSs (anionic chlorin e_6 and Al–phthalocyanine) in relation to the microorganism *E. coli* K-12 are presented in Table 4.

The higher phototoxicity (low concentrations) of the metalloporphyrins Zn–TBut4PyP and Zn–TAll4PyP is apparently due to the nature of the central metal atom, the presence of hydrophobic functional groups and high quantum yields of singlet oxygen (Table 1). The studied cationic pyridylporphyrins/metalloporphyrins exhibit 1000 times higher photodynamic activity than the anionic photosensitizers chlorin e_6 and Al–phthalocyanine used as controls.

Thus, our data confirm the hypothesis that the main factor of photoinactivation is the presence of positively charged groups in the PS molecule, which enter into electrostatic interaction with negative groups on the cell wall surface.[33,65] The final binding of PS to cells is regulated by the hydrophobicity of functional groups, which promotes strong association with the cytoplasmic membrane.[3]

3.2.3. *Study of photodynamic inactivation of various Gram negative and Gram positive bacteria by the cationic metalloporphyrin Zn–TBut4PyP*

Next, we studied the photodynamic inactivation of various Gram negative (*E. coli* K-12, *Salmonella typhimurium* G-38) and Gram positive (*S. aureus*,

Table 3. Survival (%) of *E. coli* K-12 microorganism after photoinactivation with cationic pyridylporphyrins/metalloporphyrins.

	Concentrations of PSs (μg/mL)					
PSs	0 (Control)	0.1	1	2	5	10
H_2TOE4PyP	100 ± 3.4	95.8 ± 7.2	48.3 ± 6.7	37.3 ± 7.8	15.4 ± 8.7	0.2 ± 0.1
H_2TAll4PyP	99.3 ± 4.1	82.4 ± 6.5	38.1 ± 7.2	9.4 ± 5.5	0.3 ± 0.05	0
H_2TBut4PyP	98.5 ± 5.9	82.1 ± 5.9	35.2 ± 5.6	7.1 ± 4.7	0.2 ± 0.18	0
Zn–TOE4PyP	98.4 ± 7.2	79.8 ± 7.2	32.4 ± 6.8	3.2 ± 2.6	0	0
Zn–TAll4PyP	98.2 ± 2.9	45.7 ± 5.9	13.2 ± 7.9	0.1 ± 0.01	0	0
Zn–TBut4PyP	99.5 ± 5.1	41.3 ± 6.4	10.3 ± 4.5	0	0	0

Notes: Initial cell titer: 1×10^9 CFU/mL; irradiation for 30 min at a radiation power density of 30 mW/cm^2; control: microorganisms without the addition of PSs; $p < 0.05$; and $n = 5$.

Table 4. Photodynamic activity of photosensitizers in relation to the microorganism *E. coli* K-12.

Photosensitizers	MBC (μg/mL)
Chlorin e_6	$>10,000$
Al–phthalocyanin	$>10,000$
H_2TOE4PyP	10.3 ± 0.25
H_2TBut4PyP	5.2 ± 0.40
H_2TAll4PyP	5.3 ± 0.20
Zn–TOE4PyP	5.1 ± 0.25
Zn–TBut4PyP	2 ± 0.15
Zn–TAll4PyP	2.1 ± 0.25

Notes: Initial cell titer: 1×10^9CFU/mL; irradiation for 30 min at a radiation power density of 30 mW/cm^2; control: microorganisms without the addition of PSs; $p < 0.05$; and $n = 5$.

S. epidermidis) bacteria by cationic metalloporphyrin Zn–TBut4PyP. These results are shown in Fig. 6.

As can be seen from Fig. 6, the metalloporphyrin Zn–TBut4PyP effectively photoinactivates both Gram positive and Gram negative bacteria at very low concentrations (0.7–2 μg/mL). For example, a-PDT inhibited *E.coli* K-12 bacteria growth [at a concentration of 0.7 μg/mL (5.6×10^{-7}M)] and caused a $\log_{10}$ reduction on 7.8, as seen in Fig. 6. At a concentration of 0.1μg/mL (0.8×10^{-7} M), the difference of a-PDT inhibitions of Gram negative (*E. coli* K-12 and *S. typhimurium* G-38) and Gram positive (*S. aureus* and *S. epidermidis*) bacteria growth for $\log_{10}$ is 2.9, which corresponds to a significantly strong inhibition of the growth of Gram positive bacteria (Fig. 6). In accordance with literature data, the inactivation threshold for Gram positive bacteria was several times lower than for Gram negative bacteria.[24,32,66]

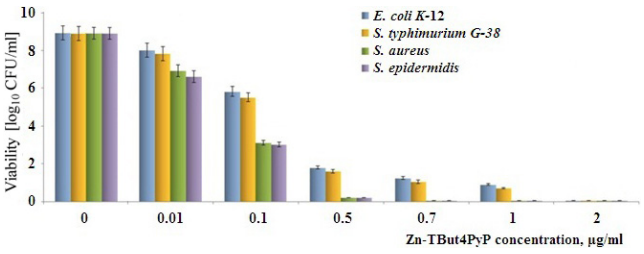


Fig. 6. Survival of Gram negative and Gram positive bacteria after photoinactivation with Zn–TBut4PyP.

3.2.4. *Study of photodynamic inactivation of methicillin-resistant pathogens by cationic pyridylporphyrins/metalloporphyrins*

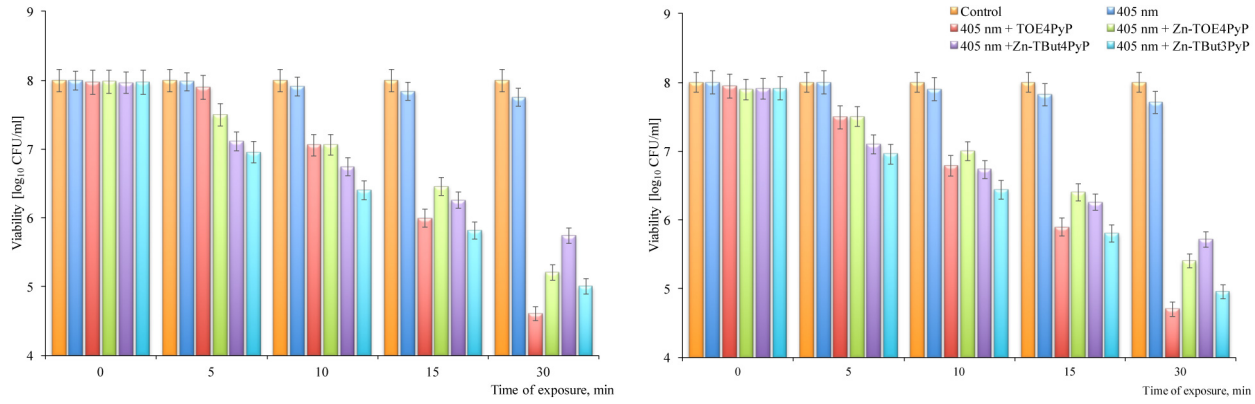
Present time is characterized by the excessive and incorrect use of antibiotics for the prevention and self-treatment, which in turn leads to the emergence of new antibiotic-resistant strains of bacteria.[67–69] Despite the search and implementation of new methods of fighting nosocomial microbes, the problem of nosocomial infections remains one of the most acute in modern conditions, gaining increasing medical and social significance. Methicillin-resistant *S. aureus* is one of the most important pathogens causing severe community- and healthcare-associated infections.[69–73]

In modern medicine, an antimicrobial photodynamic effect is an alternative way to combat diseases caused by both sensitive and antibiotic-resistant bacteria.[4,74–77,81] All porphyrin compounds studied in this work had the maximum absorption in the violet–green region of the spectrum (see Figs. 3 and 5), which made it possible to assume their high photodynamic activity against the antibiotic-resistant strain of *S. aureus* when irradiated with LED light at a wavelength of 405 nm with the band from 400 nm to 420 nm.[54,82]

To study the photodynamic activity of four cationic pyridylporphyrins/metalloporphyrins (H_2TOE4PyP, Zn–TOE4PyP, Zn–TBut4PyP and Zn–TBut3PyP), we carried out experiments using a light-emitting diode with a peak emission of 405 nm and a power density of 70 mW/cm^2, based on the scheme described earlier.[54] The concentrations of pyridylporphyrins of 0.1 μg/mL and 0.01 μg/mL (from 10^{-7} M to 10^{-8} M) were used in the work.

Violet LED light (405 nm) did not affect the growth of the two *S. aureus* strains tested. After 30 min of exposure, the survival rate remained within (7.7–8)$\log_{10}$CFU (Fig. 7).

It was of interest to evaluate the photodynamic effect using porphyrin compounds at a concentration of 0.1 μg/mL. After 10 min of irradiation, a decrease in the bacterial population of MSSA was noted by 0.1$\log_{10}$CFU after treatment with H_2TOE4PyP, by 0.5$\log_{10}$CFU after treatment with Zn–TOE4PyP, by 0.9$\log_{10}$CFU after treatment with Zn–TBut4PyP and by 1$\log_{10}$CFU after treatment with Zn–TBut3PyP. An increase in the irradiation time to 30 min led to the death of bacterial



(a) (b)

Notes: The initial cell titer is 1×10^8 CFU/lmL. The Control: bacteria suspension without the addition of PSs. Also, $p < 0.05$; and $n = 5$.

Fig. 7. Influence of porphyrin compounds at a concentration of 0.1 μg/mL ($0.8 \times 10^{-7} - 1.06 \times 10^{-7}$ M) on the efficiency of photodynamic exposure using violet illumination (405 nm, 70 mW/cm^2) on *S. aureus* cells: (A) methicillin-sensitive strain and (B) methicillin-resistant strain.

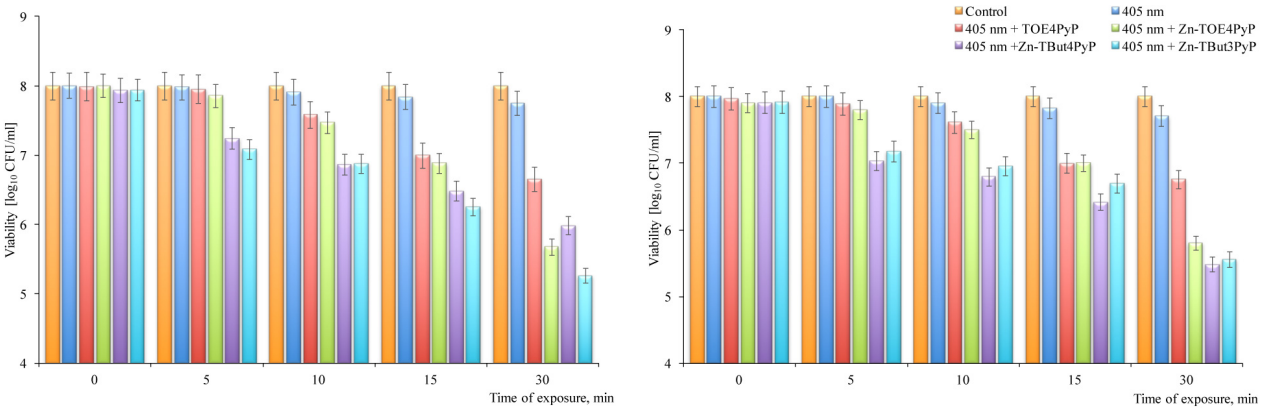
cells of this strain by (2.3–3.4)$\log_{10}$CFU. The maximum decrease in survival (by 3.4$\log_{10}$CFU) was demonstrated by the sample containing H_2TOE4PyP and Zn–TBut4PyP (Fig. 7).

Photoeffect on MRSA cells incubated with porphyrin compounds was similar in efficiency. Irradiation for 30 min caused a decrease in survival by 3.3$\log_{10}$CFU when using H_2TOE4PyP, by 2.6$\log_{10}$CFU when using Zn–TOE4PyP, by 2.3$\log_{10}$CFU when using Zn–TBut4PyP and by 3$\log_{10}$CFU when using Zn–TBut3PyP.

The use of solutions of porphyrins diluted to a concentration of 0.01 μg/mL ($\sim 10^{-8}$ M) showed that their photosensitizing properties remain quite high.

Short-term (5–10 min) LED illumination of bacterial cells incubated with porphyrin compounds caused a decrease in the CFU number by (0.1–0.4) $\log_{10}$CFU when using H_2TOE4PyP, by (0.2–0.5) $\log_{10}$CFU when using Zn–TOE4PyP, by (1–1.2) $\log_{10}$CFU when using Zn–TBut4PyP and by (0.8–1)$\log_{10}$CFU when using Zn–TBut3PyP (Fig. 8).

Samples of H_2TOE4PyP and Zn–TOE4PyP at a concentration of 0.01 μg/mL (1.06×10^{-8} M and 1×10^{-8} M, respectively) did not show a pronounced bactericidal effect after 30 min of illumination [(1.2–1.3)$\log_{10}$CFU suppression of the numbers of two studied strains]. Samples Zn–TBut4PyP and Zn–TBut3PyP in combination with



(a) (b)

Fig. 8. Influence of porphyrin compounds at a concentration of 0.01 μg/mL (0.8×10^{-8}–1.06×10^{-8} M) on the efficiency of photodynamic exposure using violet illumination (405 nm, 70 mW/cm^2) on *S. aureus* cells: (A) methicillin-sensitive strain and (B) methicillin-resistant strain.

violet illumination caused a decrease by the amount of (2.5–2.7)$\log_{10}$CFU after 30 min of exposure (Fig. 8).

Comparing the results, we can conclude that the metalloporphyrins Zn–TBut3PyP and Zn–TBut4-PyP are more promising for APDT; stable suppression of cell viability of methicillin-sensitive and methicillin-resistant strains by (2.5–3)$\log_{10}$CFU was detected after 30 min of illumination with a violet LED with a wavelength of 405 nm regardless of the concentration of porphyrin compounds used.

The cytotoxic effect of singlet oxygen and other highly active radicals formed locally upon absorption of light of a certain wavelength and power density by a photosensitizer determines the effectiveness of the APDT and directly depends on the choice of radiation type and PS.[3,5,12,61,75,82–91]

Numerous studies show that exposure of microorganisms to blue light leads to a significant decrease in their numbers.[78,79] UV–violet illumination (360–405 nm) activates endogenous or exogenous photosensitizers and involves them in a photochemical reaction. Such illumination is mainly used in dentistry, ophthalmology and dermatology due to the shallow (less than 1 mm) penetration into tissues.[79,80,82,85,92] For a number of tasks, for example, suppression of bacterial flora on large surfaces (skin, wound surfaces, surgical field, etc.), LED sources are the most optimal and cost-effective.[82,86]

The effectiveness of blue light is greatly enhanced by the use of exogenous photosensitizers, including porphyrin compounds. Among porphyrins, cationic porphyrins are most often studied as PSs, which, unlike anionic ones, do not form aggregates in solution and remain in monomeric form over the entire concentration range usually used in experiments.[21,25,30,50,80,81] This property of them greatly facilitates the processing and interpretation of experimental data.

The location of purulent microorganisms on the surface of the skin and mucous membranes of the human body makes it possible to use a combination of porphyrin compounds and low-power violet illumination for effective APDT. If microorganisms infect deep layers (up to 5–7 mm), it is possible to use optical clearing agents[93] to penetrate illumination to target cells. The coincidence of the absorption spectra of porphyrins as exogenous photosensitizers and endogenous carotenoids/flavonoids can provide a high level of ROS[5–7,12,93] production during *in-vivo* experiments and therapy.

4. Summary

For many years throughout the development and application of APDT, porphyrin compounds have attracted and continue to attract the attention of researchers as photosensitizers with well-controlled photoactive properties. Each of the listed works devoted to APDT [bacteria (*P. aeruginosa*,[82,91] *P. mirabilis*,[82] *S. aureus*,[5–7,72,84–86] *E. coli*,[23,29–31,35,86] *Mycobacteria sp.*,[40] etc.), fungi,[5–7,16] protozoa[19,20,90] and viruses[8,18]] contribute to the understanding of the antipathogenic activity of porphyrins. In this work, four pyridylporphyrins and their derivatives based on Zn were studied in detail, including their optical properties and the efficiency of singlet oxygen production.

The effectiveness of APDT using meso-substituted cationic pyridylporphyrins/metalloporphyrins was tested using Gram positive (*S. aureus*, *S. epidermidis*) and Gram negative (*E. coli*, *S. typhimurium*) microorganisms. It was found that Zn-containing complexes of pyridylporphyrins exhibit higher phototoxicity compared to metal-free pyridylporphyrins.

The data show that the high antibacterial activity of the studied cationic porphyrins/metalloporphyrins depends on four factors: (1) the presence of a positive charge in the porphyrin macrocycle (+4); (2) the presence of a central metal atom (Zn^{2+}); (3) the presence of hydrophobic peripheral functional groups; and (4) high values of singlet oxygen quantum yields.

As shown by a number of studies, APDT is able to enhance the effect of antibiotic drugs,[87–92] thus ensuring the complete destruction of the target microorganism. The results of this work indicate that meso-substituted cationic pyridylporphyrins/metalloporphyrins can find wider application in photoinactivation of bacteria than those usually used in anionic or neutral PS. The most promising application of these compounds may be the photo-induced destruction of antibiotic-resistant strains of clinically significant microorganisms. Studies have shown that in combination with violet (405 nm) LED radiation, Zn–TBut4PyP at a concentration of 0.1 μg/mL (0.8×10^{-7} M) caused the death of 3$\log_{10}$CFU of the population of methicillin-resistant *S. aureus*.

Conflict of Interest

The authors declare no conflicts of interest.

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