ORIGINAL ARTICLES

STUDY OF ACCUMULATION OF WATER-SOLUBLE ASYMMETRIC CATIONIC PORPHYRINS IN GRAM-POSITIVE WOUND INFECTION PATHOGENS DURING PHOTODYNAMIC INACTIVATION

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Abstract

The paper presents the results of a study on the accumulation of three different compounds of water-soluble asymmetric cationic porphyrins by the bacteria *S. aureus*, *S. epidermidis, S. haemolyticus* and *E. faecalis* using flow cytofluorimetry and fluorescence microscopy. The studied microorganisms were a sample (n=4) of isolates from biomaterial (wound discharge) from patients with wound infections (burn wound, trophic ulcer, infection of the surgical area, etc.). The tested strains showed resistance to 1-7 antibiotics, two strains were carriers of the *mecA* gene. Porphyrins containing heterocyclic fragments (benzoxazole, N-methyl benzimidazole, and benzothiazole residues) on the periphery of the porphyrin cycle can accumulate in bacterial cells to varying degrees: porphyrin with N-methyl benzimidazole penetrates bacteria to a greater extent, and the fluorescence signal is most intense for S. aureus and E. faecalis after incubation with this species. connections. There is some heterogeneity in the bacterial cell population with respect to the ability to accumulate porphyrins, and the presence of bacterial lysis has been proven. S aureus after incubation with S-porphyrin and subsequent photodynamic inactivation under the influence of light. The data obtained determine the prospects for further study of compounds and determination of their bactericidal potential.

Keywords: antimicrobial photodynamic inactivation, photochemistry, porphyrin, antibiotic resistance, wound infection, accumulation.

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ИЗУЧЕНИЕ НАКОПЛЕНИЯ ВОДОРАСТВОРИМЫХ НЕСИММЕТРИЧНЫХ КАТИОННЫХ ПОРФИРИНОВ В ГРАМПОЛОЖИТЕЛЬНЫХ ВОЗБУДИТЕЛЯХ РАНЕВЫХ ИНФЕКЦИЙ ПРИ ФОТОДИНАМИЧЕСКОЙ ИНАКТИВАЦИИ

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Резюме

В работе представлены результаты исследования по изучению накоплении трёх разных соединений водорастворимых несимметричных катионных порфиринов бактериями *S. aureus*, *S. epidermidis*, *S. haemolyticus* и *E. faecalis* с помощью проточной цитофлуориметрии и флуоресцентной микроскопии. Исследуемые микроорганизмы – выборка (n=4) изолятов из биоматериала (раневое отделяемое) от пациентов с раневыми инфекциями (ожоговая рана, трофическая язва, инфекция области хирургического вмешательства и др.). Тестируемые штаммы проявляли устойчивость к 1-7 антибиотикам, два штамма были носителями гена *mecA*. Порфирины, содержащие на периферии порфиринового цикла гетероциклические фрагменты (остатки бензоксазола, N-метил бензимидазола и бензотиазола), в разной степени способны накапливаться в бактериальных клетках: порфирин с N-метил бензимидазолом в большей степени проникает в клетки бактерий, и сигнал флуоресценции наибольшей интенсивности наблюдается для *S. aureus* и *E. faecalis* после инкубации с данным видом соединения. Наблюдается некоторая гетерогенность популяции бактериальных клеток в отношении способности накапливать порфирины, доказано наличие лизиса бактерий *S. aureus* после инкубации с фотосенсибилизатором и последующей фотодинамической инактивацией под действием света. Полученные данные определяют перспективы для дальнейшего изучения соединений и определения их бактерицидного потенциала.

Ключевые слова: антимикробная фотодинамическая инактивация, фотохимия, порфирин, антибиотикорезистентность, раневая инфекция, накопление.

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Introduction

With the increasing resistance of microorganisms to antibiotics [1,2], there is a growing interest worldwide in alternative methods and biocides capable of overcoming the polyresistance of leading infectious agents. One promising area is antimicrobial photodynamic inactivation (PDI), based on photochemical reactions in which the main role is played by reactive oxygen species produced by molecules of a non-toxic dye or photosensitizer in the presence of low-intensity visible light to destroy microbial cells [3,4].

Antimicrobial photodynamic therapy, being a particular option of photodynamic therapy (PDT), has found its place in the fight against infectious diseases in various areas of clinical medicine: dentistry [5,6], dermatology [7-9], gynecology [10,11], urology [12], otolaryngology [13,14], etc.

In addition, in recent years, clinical interest has increased and serious scientific prerequisites for the use of PDI for the treatment of infected wounds of the skin and soft tissues have emerged. From a therapeutic point of view, this is determined by a number of advantages for the patient like high precision and selectivity of local action, low invasiveness, atraumatic nature, and additional effects in the form of stimulation of regeneration processes with acceleration of healing. From a microbiological point of view, it is important that this type of therapy can be used repeatedly with the same photosensitizer without any apparent risk of developing resistance, since at the moment there are no unambiguous reports that microorganisms exhibit resistance, in a certain sense of the word, to PDI. Moreover, although authors from different research teams [15-17] observed the effect of decreasing the susceptibility of the population of archival and clinical strains to sublethal doses of irradiation after several cycles of photoinactivation [15-17], this cannot be interpreted as the development of resistance to PDI, since when using more stringent experimental conditions (increasing the concentration of the photosensitizer and/or increasing the number of light doses), bacteria were destroyed [18].

Limitations in the active use of the antimicrobial PDI method in medical practice are associated with a shortage of non-toxic drugs with high bactericidal efficacy against planktonic and biofilm forms of microorganisms.

Studying the properties of experimental photosensitizers to find compounds with optimal antimicrobial activity that participate in the fight against resistant pathogens is a priority task in clinical, microbiological and epidemiological terms.

It is known that anionic, neutral and cationic photosensitizers are active against gram-positive bacteria, while only cationic ones have an effect on gram-negative bacteria [19]. Thus, to expand the spectrum of bactericidal action, it is more appropriate to use cationic photoinactivators.

The potential of water-soluble asymmetric cationic porphyrins is determined by the efficient production of singlet oxygen and the advantage of the possibility of chemical modification of peripheral substituents for selective binding to biotargets of microbial cells [19-21]. However, to understand the bactericidal potential of these compounds in an *in vitro* experiment, additional studies are needed, including studies of the

accumulation of the photosensitizer in microbial cells of clinical strains of microorganisms.

Studying the features of photochemical reactions in a population of antibiotic-resistant clinical strains of pathogens brings our experience closer to conducting preclinical studies *in vivo* and clinical trials.

It is extremely important to note that the scientific and practical task is not only to search for new compounds or modify existing molecules to maximize the quantum yield of singlet oxygen in *in vitro* tests, but also to study the molecular bonds inside microbial cells and the factors influencing differences in the susceptibility of microbes to photoinactivation [22]. The search for mechanisms, phenotypic and genotypic features underlying various microbial responses to photodynamic inactivation is an important problem of antimicrobial PDI, since even a population of one bacterial agent existing in one infection site is heterogeneous in its properties. Moreover subpopulations of the pathogen can exhibit different susceptibility to antimicrobial factors, including PDI, which can be determined by a number of factors: persistent cells are less susceptible to antimicrobial PDI than fast-growing ones, have a higher potential for producing antioxidant enzymes, etc. One of the main conditions determining the antimicrobial effect of PDI is the ability of cells to accumulate a photosensitizer.

The aim of the study is to study the features of porphyrin accumulation in cells of gram-positive antibiotic-resistant bacteria during PDI *in vitro*.

Materials and methods

This study is planned as an exploratory, comprehensive and multi-stage one. In the first stages of the work, a group of researchers from the G. A. Krestov Institute of Solution Chemistry of the Russian Academy of Sciences (Ivanovo) synthesized asymmetric water-soluble porphyrins containing heterocyclic fragments (benzoxazole, N-methyl benzimidazole and benzothiazole residues) [23] on the periphery of the porphyrin cycle using the C—H activation method. The experiments conducted on direct and reverse spectrophotometric titration of the interaction (binding) of albumin with monoheteryl-substituted porphyrins [23] allowed us to assume that porphyrins, when interacting with a bacterial cell, target surface proteins and the genetic material of microorganisms. These data served as the basis for further work.

This study presents the following research results: data on the accumulation of porphyrins *in vitro* in gram-positive bacteria using flow cytofluorometry and fluorescence microscopy.

Experimental studies were carried out at three bases:

1. synthesis of chemical compounds: Federal State Budgetary Scientific Institution G.A. Krestov Institute of Solution Chemistry of the Russian Academy of Sciences, Ivanovo;
2. microbiological studies on the isolation, identification and study of antibiotic resistance of microorganisms: bacteriological laboratory of the University Clinic of the Federal State Budgetary Educational Institution of Higher Education "Priority Medical University" of the Ministry of Health of the Russian Federation, Nizhny Novgorod;
3. studies on the accumulation of a photosensitizer in bacterial cells: Research Center of Molecular Biology and Biomedicine of the Federal State Autonomous Educational Institution of Higher Education "National Research Nizhny Novgorod State University named after N.I. Lobachevsky", Nizhny Novgorod.

In a series of laboratory experiments, the main objects of study were three different compounds of monoheteryl-substituted porphyrins and strains of gram-positive bacteria.

The porphyrins studied were:

1) 5-[4′-(1′′,3′′-benzothiazol-2′′-yl) phenyl]-10,15,20-tris(N-methylpyridin-3′-yl) porphyrin triiodide (S-por). PBS (phosphate buffered saline) (7.4) M=1176.73 C=1*10-5 mol/l;
2) 5-[4′-(1′′,3′′-benzoxazol-2′′-yl) phenyl]-10,15,20-tris(N-methylpyridin-3′-yl) porphyrin triiodide (O-por). PBS (7.4) M=1160.67 C=1*10-5 mol/l;
3) 5-[4-(N-methyl-1′′,3′′-benzimidazole-2′′-yl) phenyl]-10,15,20-tris(N-methyl-pyridin-3′-yl)-porphyrin triiodide (N-por). PBS (7.4) M=1173.74 C=1*10-5 mol/l.

The studied microorganisms are a sample (n=85) of microorganism isolates from biomaterial (wound discharge) of patients with wound infections. The experiment included clinical material from patients of the Burn Center and the Institute of Traumatology and Orthopedics of the University Clinic of the Federal State Budgetary Educational Institution of Higher Education "Privolzhsky Research Medical University (PRMU)" of the Ministry of Health of the Russian Federation with various purulent-septic infections of the skin and soft tissues (burn wound, trophic ulcer, surgical site infection, etc.). Species composition of the collection:

To study the features of porphyrin accumulation in bacterial cells, one strain of *S. aureus, S. epidermidis, S. haemolyticus,* and *E. faecalis.* were selected from the general population.

Species identification of microorganisms was carried out by MALDI-TOF mass spectrometry on the appropriate equipment (MALDI-TOF MS (Germany) and MALDI-TOF AUTOF MS1000 (Autobio, China)). The study of susceptibility to antibacterial drugs was carried out on a Vitek 2 bacteriological analyzer (France), with subsequent interpretation according to the adopted

rules of EUCAST (European Committee on Antimicrobial Susceptibility Testing) [24]. The prevalence of resistance genes was determined using the AmpliSens MDR MBL-FL and Litekh reagent kits (Russia) for the isolation of resistance genes by PCR with hybridization-fluorescence detection of amplification products in real time. The *mecA* gene was detected in staphylococci. The properties of the isolated strains (antibiotic resistance) were described using the version 2023 of the WHONET program.

To obtain data on the accumulation of porphyrins in bacterial cells, the following steps were performed:

1.1 Preparation of bacterial cell culture. An overnight culture of *S. aureus, S. epidermidis, S.haemolyticus* and *E. faecalis* was grown in sterile liquid LB medium (10 g of tryptone, 10 g of NaCl, 5 g of yeast extract (all from Sigma-Aldrich, USA)) for 16-18 h at 37 °C. The resulting suspension was centrifuged (10 min, 4000g) and diluted to a concentration of $1.5x10^8$ cells/ml in sterile 0.9% NaCl solution.

1.2 Incubation of bacterial cells with porphyrins. S-, O- and N-porphyrins were dissolved in sterile PBS (pH 7.4) (10 μM) and added to the bacterial suspension ($1.5x10^7$ cells/ml). For photoinactivation, the bacterial suspension was irradiated with LED lamps (20 W power) for 15 min at room temperature.

1.3 Evaluation of absorption and accumulation of porphyrins by bacteria. To assess the absorption of the studied porphyrins by bacteria after incubation, the bacteria were washed twice with sterile PBS (10 min, 4000 g, 4 °C) and the resulting suspension was analyzed using a Cytoflex S flow cytometer (Beckman Coulter, USA) and CytExpert 1.2.10.0 software (Beckman Coulter, USA).

The accumulation of porphyrins was estimated based on the average values of the fluorescence signal (mean fluorescence intensity, MFI), measured in relative units and recorded for *S. aureus, S. epidermidis, S. haemolyticus* and *E. faecalis bacteria* after treatment with S-, O- and N-porphyrins after 15 minutes of light irradiation. For fluorescence analysis using microscopy, a bacterial suspension ($1.5x10^7$ cells/ml) after incubation with 10 μM solutions of S-, O-, and N-porphyrins was centrifuged (5 min, 4000 g, 4 °C). The pellet was resuspended in sterile PBS (pH 7.4) and applied to glass slides.

A drop containing the bacterial suspension was dried in air, fixed in a burner flame, then placed in Faramount Mounting Medium (Agilent, USA) and covered with a coverslip. The study was carried out on an LSM 800 setup (Carl Zeiss, Germany) using ZEISS Axio Vert.A1 software (Carl Zeiss, Germany). The accumulation of porphyrins was assessed by the presence of fluorescence (excited using a 488 nm laser, the fluorescent signal was detected in the wavelength range of 640-740 nm using appropriate optical filters).

Statistical data processing was performed in the R environment (Rstudio 1.1.463), and GraphPad Prism 8.4.3 software (GraphPad Software, USA) was used for data visualization. Two-way analysis of variance was used to compare mean fluorescent signal (MFI) values. The level of statistical significance of differences in hypothesis testing was chosen at $p \leq 0.05$.

Results and discussion

The tested strains (n=4) showed resistance to 1-7 antibiotics. The resistance profiles of the selected cultures are presented in Table 1. Two strains (*S.haemolyticus* 525, *S.epidermidis* 7) were resistant to cefoxitin, which is an alarming microbiological and clinical sign, since, according to the EUCAST guidelines, resistance to this antibiotic is a sign of resistance to the entire group of penicillins. At the same time, these strains were carriers of the *mecA* gene.

In an *in vitro* experiment to study the accumulation of porphyrins by bacterial cells of clinical strains of *S. aureus*, *E. faecalis*, *S. epidermidis* and *S. haemolyticus* using

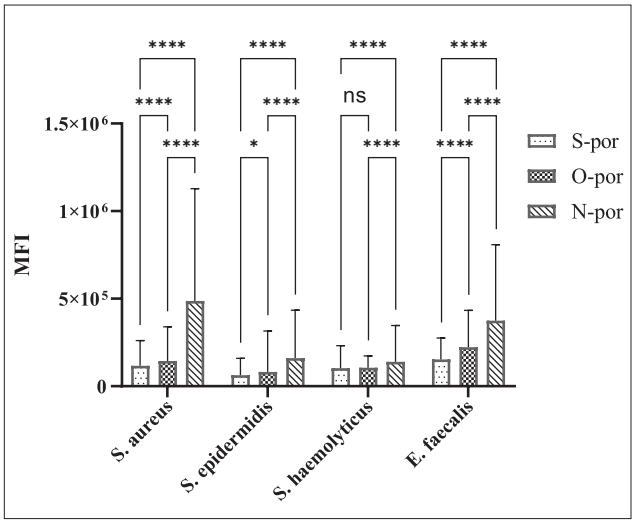
Таблица 1
Профили антибиотикорезистентности клинических штаммов

Table 1
Profiles of antibiotic resistance of clinical strains

Микроорганизм и идентификационный номер по лабораторному журналу / результаты ПЦР The microorganism and the identification number according to the laboratory journal / PCR results	Профиль резистентности Resistance profile
S.aureus 229 / бета-лактамазы +	B_--__PL_-___H__
S.epidermidis 7 / *mecA*+	-_--XGPLN-_Z_H_-
S.haemolyticus 525 / *mecA*+	----X----------
E. faecalis 2025	--_- G-_NM_-_---

В столбце «Профиль резистентности» использована знаковая система: «буква» – резистентность или промежуточная чувствительность к определенному антибиотику; «_» – чувствительность; «-» – чувствительность не определялась. Расшифровка буквенных кодов в профилях резистентности: B – бензилпенициллин; R – рифампицин; C – цефтриаксон; F – цефотаксим; X – цефокситин; G – гентамицин; P – ципрофлоксацин; L – левофлоксацин; N – клиндамицин; M – линкомицин; E – эритромицин; Z – линезолид; V – ванкомицин; H – хлорамфеникол; T – тетрациклин; Y – тигециклин. «mecA+» – у штамма обнаружен соответствующий ген.

The column "resistance profile" uses a sign system: "letter" – resistance or intermediate sensitivity to a particular antibiotic; "_" – sensitivity; "-" – sensitivity to a particular antibiotic. Interpretation of letter codes in resistance profiles: B – benzylpenicillin; R – rifampicin; c – ceftriaxone; F – cefotaxime; X – cefoxitin; G – gentamicin; P – vrofloxacin; L – levofloxacin; N – clindamycin; M – lincomycin; e – erythromycin; Z – linezolid; V – vancomycin; H – chloramphenicol; T – tetracycline; Y – tigrecycline. XR – resistance to cefoxitin. "mecA+" – the corresponding gene was found in the strain.



cytofluorimetric analysis, it was found that N-porphyrin accumulates in bacterial cells to a greater extent than S- and O-porphyrins, as evidenced by the increasing mean fluorescence intensity (MFI) in the series S-porphyrin > O-porphyrin > N-porphyrin (Fig. 1).

Рис. 1. Значения среднего флуоресцентного сигнала (MFI), регистрируемого для бактерий *S. aureus, S. epidermidis, S. haemolyticus* и *E. faecalis* после обработки S-, O- и N-порфиринами через 15 минут облучения светом (статистика: двухфакторный дисперсионный анализ, *– p<0,05; ****– p<0,0001, данные отображают среднее и стандартное отклонение).

Fig. 1. The values of the average fluorescent signal (MFI) recorded for *S. aureus, S. epidermidis, S. haemolyticus* and *E. faecalis* bacteria after treatment with S-, O-, and N-porphyrins after 15 minutes of light exposure (statistics: two–factor analysis of variance, *– p<0.05; ****– p<0.0001, the data shows the mean and standard deviation).

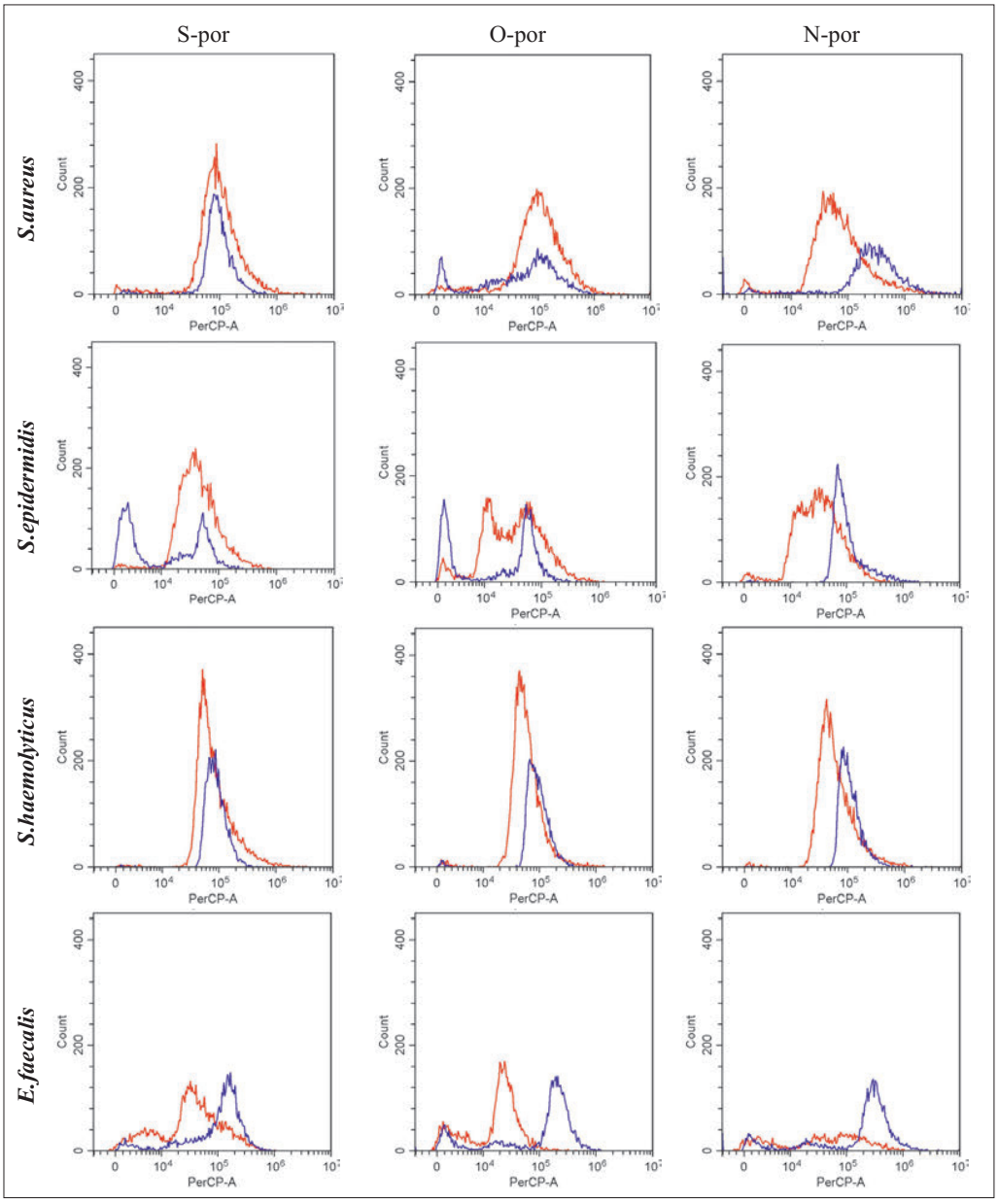


Рис. 2. Репрезентативные гистограммы флуоресценции бактерий *S. aureus, S. epidermidis, S. haemolyticus* и *E. faecalis* после обработки S-, O- и N-порфиринами: ■ – до обработки светом, ■ – после обработки светом 15 мин.

Fig. 2. Representative histograms of fluorescence of *S. aureus, S. epidermidis, S. haemolyticus* and *E. faecalis* bacteria after treatment with S-, O- and N-porphyrins: ■ –before light treatment, ■ – after light treatment for 15 min.

Moreover, for *S. aureus* and *E. faecalis*, the mean fluorescence intensities were higher than for *S. epidermidis* and *S. haemolyticus*. Also, for *S. epidermidis* and *S. haemolyticus*, the difference between S- and O-porphyrin accumulation was not as significant (5% difference in significance for *S. epidermidis* and no difference for *S. haemolyticus*) than *for S. aureus* and *E. faecalis*.

Representative graphs obtained during cytofluorometric analysis are shown in Fig. 2. The graphs demonstrate that the fluorescence peak is shifted along the PerCP-A axis to the right for N-porphyrin to a greater extent than for S- and O-porphyrin. Moreover, in accordance with Fig. 1, in Fig. 2 it is observed that this shift is more pronounced for *S. aureus* and *E. faecalis*, which is probably due to the greater accumulation of N-porphyrin by these cells.

For *S. aureus, S. epidermidis* and *S. haemolyticus*, the graphs demonstrate a decrease in the peak height and/or the appearance of an additional peak, which probably characterizes the presence of cells that do not accumulate S- and O-porphyrins or accumulate them to a lesser extent.

Using fluorescence microscopy, data were obtained confirming the fact of accumulation of the porphyrins used in the work by *S. aureus*, *S. epidermidis, S. haemolyticus* and *E. faecalis* (Fig. 3).

We also observed that some cells accumulate porphyrins to a lesser extent than the main population (Fig. 4a), which confirms the data obtained using flow cytofluorimetry on the presence of cells with lower fluorescence intensity or its absence in the population. In addition, it was shown that coincubation with porphyrins followed by photoinactivation leads to cell lysis (Fig. 4b), which determines the bactericidal potential of the studied compounds.

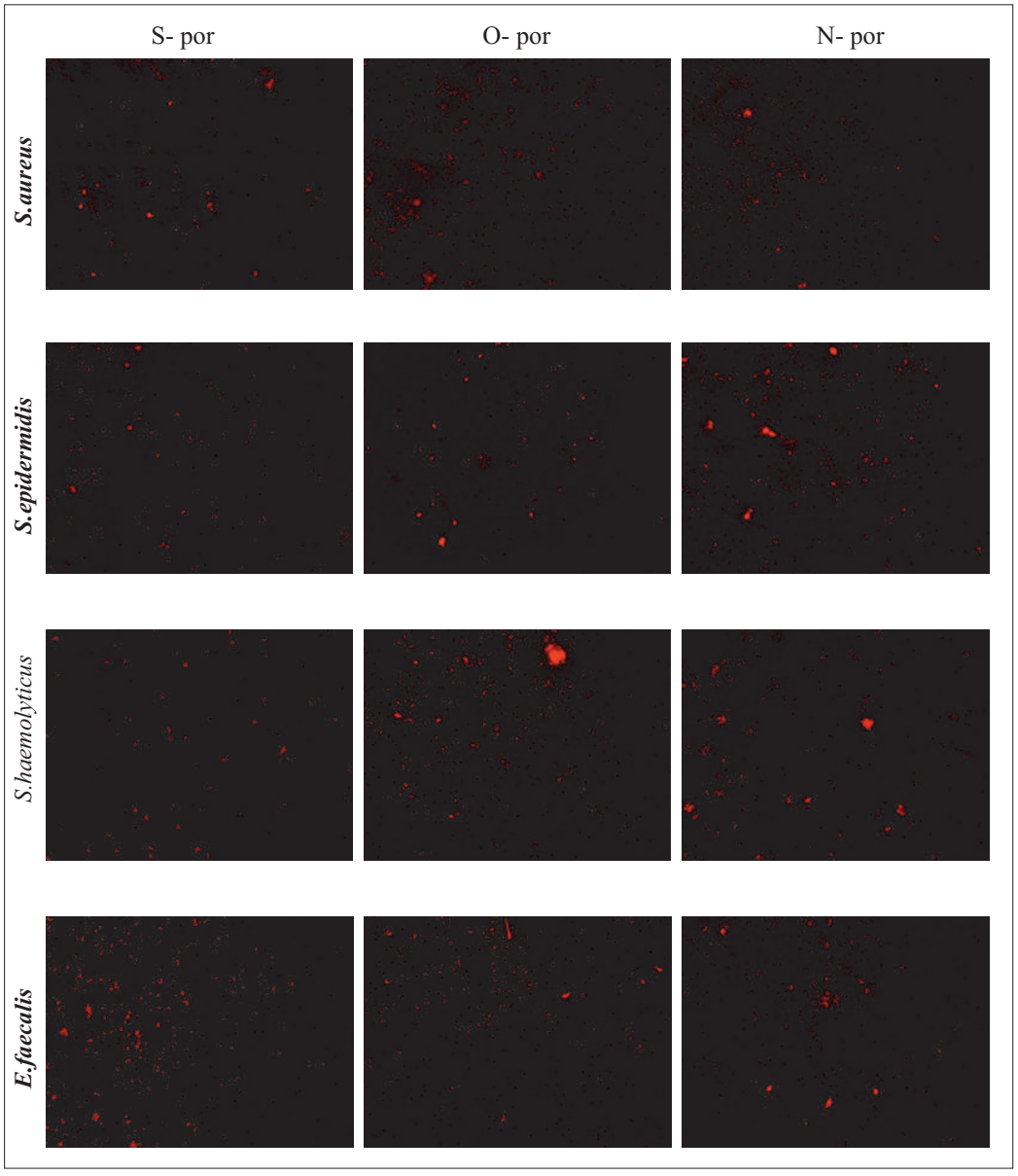


Рис. 3. Репрезентативные микрофотографии флуоресценции бактерий *S. aureus, S. epidermidis, S. haemolyticus* и *E. faecalis* после обработки S-, O- и N-порфиринами (данные получены с помощью микроскопа ZEISS Axio Vert.A1 (Carl Zeiss, Германия), увеличение 40x).

Fig. 3. Representative micrographs of the fluorescence of *S. aureus, S. epidermidis, S. haemolyticus* and *E. faecalis* bacteria after treatment with S-, O- and N-porphyrins (data obtained using a ZEISS Axio Vert. A1 microscope (Carl Zeiss, Germany), magnification 40x).

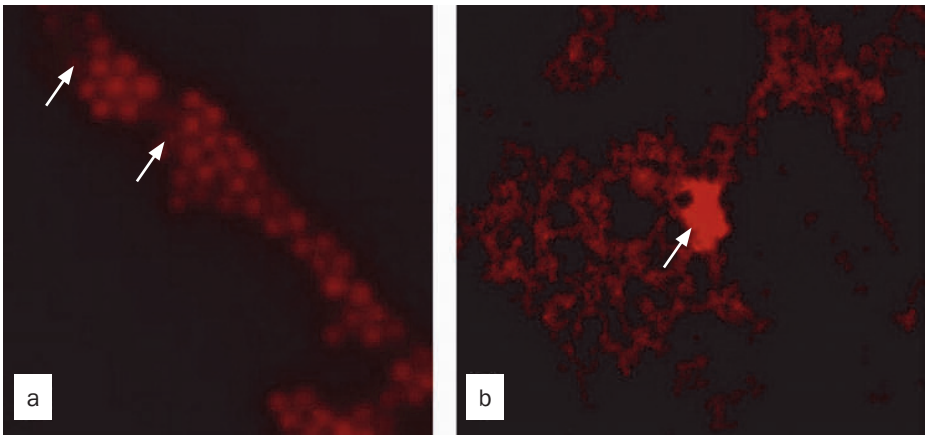


Рис. 4. Репрезентативные микрофотографии флуоресценции бактерий *S. aureus* после обработки S-порфирином, демонстрирующие (а) различия в интенсивности флуоресценции для отдельных бактерий (увеличение 100х), (б) наличие лизиса бактерий (увеличение 40х).
Fig. 4. Representative micrographs of *S. aureus* fluorescence after S-porphyrin treatment, showing (a) differences in fluorescence intensity for individual bacteria (magnification of 100x), (b) the presence of bacterial lysis (magnification of 40x).

In experimental conditions, evidence was obtained that the population of antibiotic-resistant microorganisms – causative agents of wound infections, is heterogeneous in its ability to accumulate porphyrin, which means that the bactericidal effect can also vary. This is important for further research and determining the basic parameters of antimicrobial PDI using porphyrins in patients with infection. Namely, it is necessary to more carefully approach the development of exposure conditions (volume and concentration of the photosensitizer, irradiation time), since it is possible for some part (subpopulation) of microorganisms to survive after irradiation.

Conclusion

Based on the results of the *in vitro* experiment, it was determined that asymmetrical water-soluble porphyrins containing heterocyclic fragments (benzoxazole, N-methyl benzimidazole and benzothiazole residues) on the periphery of the porphyrin cycle are able to accumulate in bacterial cells to varying degrees. The study assessed the accumulation of S-, O-, and N-porphyrins by *S. aureus, S. epidermidis, S. haemolyticus,* and *E. faecalis bacteria* using flow cytometry and fluorescence microscopy. It was confirmed that the porphyrins used were able to accumulate in bacterial cells, and a comparison of their accumulation efficiency in different types of gram-positive bacteria was performed.

It was shown that N-porphyrin penetrates bacteria to a greater extent, and the fluorescence signal of the highest intensity is observed for *S. aureus* and *E. faecalis* after incubation with this type of porphyrin. The data obtained were confirmed by fluorescence microscopy. It was also found that there is some heterogeneity in the bacterial cell population with respect to the ability to accumulate water-soluble asymmetric cationic porphyrins, and the presence of lysis of *S. aureus* bacteria after incubation with S-porphyrin and subsequent photodynamic inactivation under the light was demonstrated.

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