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STUDY OF THE POSSIBLE DEVELOPMENT OF BACTERIAL RESISTANCE TO PHOTODYNAMIC INACTIVATION

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The increasing number of pathogens resistant to antibiotics requires the development of alternative antibacterial strategies. One of the most promising and innovative approaches for the destruction of pathogens is the photodynamic inactivation (PDI) of microorganisms. In order to identify the ability of bacteria to develop resistance to PDI, studies have been conducted on strains of *Staphylococcus aureus* 62-A and *Escherichia coli* K-12. Tetracationic Zn-mesotetra-[4-N-(2`-butyl) pyridyl]porphyrin (Zn-TBut4PyP) was used as a photosensitizer (PS). After activation by visible light the ability of Zn-TBut4PyP) to develop resistance in these strains was investigated. It was shown that no resistant mutants were detected after successive 10-fold repetition of the PDI procedures of microorganisms with Zn-TBut4PyP.

Photodynamic inactivation – porphyrin derivative – pathogens – Staphylococcus aureus – Escherichia coli

Հակաբիոտիկների նկատմամբ կայուն պաթոգեն մանրէների թվի աճը պահանջում է հակաբակտերիալ այլընտրանքային մոտեցումների մշակում: Պաթոգենների ոչնչացման համար առավել խոստումնալից և նորարարական մոտեցումներից մեկը համարվում է միկրոօրգանիզմների ֆոտոդինամիկ ապաակտիվացումը (ՖԴԱ): ՖԴԱ-ի նկատմամբ բակտերիաների կայունություն զարգացնելու ունակությունը ստուգելու նպատակով իրականացվել են հետազոտություններ *Staphylococcus aureus* 62-A և *Escherichia coli* K12 շտամների վրա: Որպես ֆոտոսենսիբիլիզատոր օգտագործվել է տետրակատիոնային Zn-մեզո-տետրա[4-N-(2'-բութիլ) պիրիդիլ]պորֆիրինը (Zn-TBut4PyP): Ուսումնասիրվել է Zn-TBut4PyP-ով (տեսանելի լույսով ակտիվացումից հետո) այդ շտամների մոտ կայունություն առաջացնելու ունակությունը: Ցույց է տրվել, որ *Zn-TBut4PyP*-ով ՖԴԱ-ի գործընթացի հաջորդական 10 անգամ կրկնությունից հետո կայուն մուտանտներ չեն հայտնաբերվել:

ֆոտոդինամիկ ապաակտիվացում – պորֆիրինի ածանցյալ – պաթոգեններ – Staphylococcus aureus – Escherichia coli

Возрастающее число патогенов, устойчивых к антибиотикам, требует разработок альтернативных антибактериальных стратегий. Одним из наиболее перспективных и инновационных подходов для уничтожения патогенов является фотодинамическая инактивация (ФДИ) микроорганизмов. С целью выявления способности бактерий вызывать устойчивость к ФДИ микроорганизмов, исследования были проведены на штаммах *Staphylococcus aureus* 62-А и *Escherichia coli* К12. Тетракатионный Zn-мезотетра-[4-N-(2'-бутил) пиридил]порфирин (Zn-TBut4PyP) был использован в качестве фотосенсибилизатора (ФС). Была исследована способность Zn-TBut4PyP (после активации видимым светом) вызвать устойчивость у этих штаммов. Показано, что после последовательного 10-кратного повторения процедур ФДИ с Zn-TBut4PyP устойчивых мутантов не обнаружено.

Фотодинамическая инактивация – производный порфирина – патогенны – Staphylococcus aureus – Escherichia coli

The widespread and increasing antibiotic resistance among pathogenic bacteria has forced researchers to find efficient alternative therapeutic methods against which the bacteria are not able to easily develop resistance [4]. Photodynamic therapy could be one of such alternative strategies, which is expected to be useful in the treatment of localized infections [10, 15, 21]. The mechanism of PDI action is a multi-target damaging process in contrast to those of antibiotics, which act very specifically on a definite target. PDI of microorganisms is based on the concept that positively charged PSs can attach and/or accumulate in or at the pathogen to induce irreversible damage upon light activation of the PS [12]. This process of PDI involves the delivering light of the appropriate wavelength to the PS molecule in order to bring it to an excited singlet state, which subsequently crosses to a more stable triplet state with lower energy via intersystem crossing. The interaction between the PS excited states and endogenous oxygen in the immediate vicinity of the target cell provides cytotoxic effects due to the production of highly toxic reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$) and free radicals like hydrogen peroxide (H_2O_2), superoxide radical anion ($O_2^{\bullet-}$) and hydroxyl radical ($OH^{\bullet}$), able to irreversibly alter vital components of cells resulting in oxidative lethal damage [1, 8, 10, 11]. It should be mentioned that traditional antibiotics can also use ROS to create lethality to bacteria [6], although such ROS are generated endogenously, in contrast to PDI where the ROS are generated by PS. Another major feature of PDI as compared to typical antibiotics is that PSs can attack bacteria at multiple sites through binding to targets of extracellular or intracellular localization [12, 15]. Until now it has been questionable if bacteria can develop resistance to this peculiar kind of stress [7, 15]. In order to dispel these doubts, it is necessary to conduct multistage studies to investigate the ability of pathogenic strains to develop resistance when exposed to PDI with tetracationic Zn-TBut4PyP. For this *in vitro* study the gram-positive *S. aureus* 62-A and the gram-negative *E. coli* K-12 strains were selected. Studies of bacterial resistance to PDI are generally based on sub-lethal treatments followed by the growth of surviving colonies in repeated cycles under the same conditions [1].

Materials and methods. ***Bacterial strains and growth procedures.*** In this study the bacterial strains *E. coli* K-12 (from the collection of microorganisms of the Scientific and Production Center "Armbiotechnology" of NAS of Armenia) and *S. aureus* 62-A (from the collection of microorganisms of the A. Aleksanyan Research Institute of Epidemiology, Virology and Medical Parasitology, RA Ministry of Health) were used. Luria-Bertani (LB) medium was used as a complete liquid and solid medium [16]. Bacterial strains were grown in LB medium, at 37 °C with orbital shaking overnight. Cells were harvested by centrifugation (13,000 rpm for 10 min) and washed twice in phosphate-buffered saline (PBS), pH 7.4. The cells were then resuspended in the same buffer to a final concentration of 10^8 CFU mL^{-1}. The optical density (OD) of cell suspension was measured with a spectrophotometer Shimadzu UV-Recording Spectrophotometer UV-2100 (Japan) at a wavelength 540 nm.

Photosensitizer. The tetracationic Zn-TBut4PyP used as a PS was synthesized at the Department of Chemistry, Pharmaceutical Faculty, Yerevan State Medical University and kindly provided by Ph.D., associate professor R.K. Kazaryan [13]. The original solution of Zn-TBut4PyP was prepared in sterile distilled water and stored at room temperature in the dark until use.

Light source. Irradiation was carried out using a 50 W tungsten lamp with an irradiation range of 320-780 nm and an irradiation power density of 30 mW cm^{-2} for 30 min.

Phototoxicity assay. Samples for the study contained 0.1 ml of the PS solution of the appropriate concentration (final concentrations of 0.5, 1, 0.2, 5, 10 and 20 μg mL^{-1}) and 0.9 ml of suspension of microorganisms. Samples were incubated in the dark with appropriate concentrations of Zn-TBut4PyP at room temperature for 10 minutes, and then all samples were irradiated for 30 minutes with an irradiation power density of 30 mW cm^{-2}. Samples without exposure and with/without Zn-TBut4PyP, as well as without Zn-TBut4PyP and with irradiation served as controls.

The sensitivity of each strain was determined *in vitro* by the method of serial dilutions [18]. All experiments were performed in triplicate. After 24 h incubation at 37 °C, the number of colony forming units (CFU) of strains was counted and the minimum bactericidal concentration (MBC) was determined as the minimum concentration of Zn-TBut4PyP leading to more than 99.9 % cell killing (~3 log CFU mL^{-1}).

Multistep selection of PDI-resistant microorganisms. For each strain, single colonies that survived PDI in the presence of Zn-TBut4PyP in the concentration corresponding to the MBC was selected from the plate, inoculated in 5 ml of appropriate medium, and grown at 37 °C. After 24 h, the cells were harvested by centrifugation (13,000 rpm for 10 min) and washed twice in PBS, pH 7.4. Then the cells were suspended in the same buffer to a final concentration of 10^8 CFU mL^{-1} and the Zn-TBut4PyP in corresponding concentrations was added. Photodynamic inactivation was repeated as described above. Following this protocol, 10 PDI procedures were performed for each strain. Resistance selection was studied by determining the MBC every day.

Statistical analysis. The statistical parameters (average values, standard deviation) used in the experiments were calculated using the MS Excel. Each experiment was conducted in triplicate. The calculation was referred to untreated controls, which were neither incubated with photosensitizer nor irradiated.

Results and Discussion. The effectiveness of PDI of microorganisms with tetracationic Zn-TBut4PyP was tested against two bacteria: the gram-positive *S. aureus* 62-A and the gram-negative *E. coli* K-12 strains. The effectiveness of Zn-TBut4PyP was evaluated on the basis of determining the number of visible CFU mL^{-1} after photoinactivation and the minimum MBC was determined as the minimum concentration of Zn-TBut4PyP leading to more than 99.9 % cell killing (~3 log CFU mL^{-1}).

Photoinactivation of the *S. aureus* 62-A strain by Zn-TBut4PyP led to a decrease in viability upon irradiation for 30 min (fluence rate of 30 mW cm^{-2}) depending on the PS concentration. Zn-TBut4PyP at a concentration of 0.5 μg mL^{-1} resulted in 99.9 % (MBC) cell death of the *S. aureus* strain (fig. 1).

The growth of the tested *S. aureus* 62-A strain without PS was not affected with irradiation (light control), demonstrating that the light dose of 30 mW cm^{-2} used during the experiments does not have an antibacterial effect itself (fig. 1). At the same time, the *S. aureus* samples incubated with Zn-TBut4PyP but not irradiated (dark control) were not affected, showing that Zn-TBut4PyP in the dark does not have antibacterial effect at the concentrations used (fig. 1).

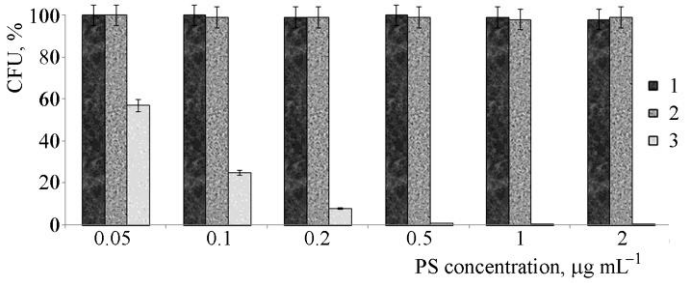

Fig. 1. Effectiveness of photoinactivation of *S. aureus* 62-A with Zn-TBut4PyP
1 – light control; 2 – dark control; 3 – photoinactivation of *S. aureus* 62-A

Irradiation of *E. coli* K-12 strain after incubation with Zn-TBut4PyP at a concentration of 1 μg mL^{-1} revealed a reduction in the number of viable colonies by more than 99.9 % (MBC) (fig. 2). As in the previous case with *S. aureus* 62-A strain, the light and dark controls showing that the viability of *E. coli* K-12 strain was neither

affected by light alone nor by the Zn-TBut4PyP in the dark with the tested concentrations (fig. 2).

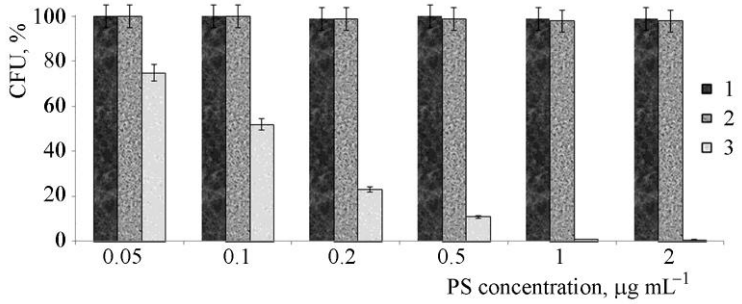

Fig. 2. Effectiveness of photoinactivation of *E. coli* K-12 with Zn-TBut4PyP
1 – light control; 2 – dark control; 3 – photoinactivation of *E. coli* K-12

In agreement with the data reported in the literature for other cationic PSs [2, 17], *S. aureus* 62-A showed high susceptibility to PDI with Zn-TBut4PyP compared to *E. coli* K-12.

To determine development of resistance to Zn-TBut4PyP with irradiation in the experimental strains used, the variation of MBCs during 10 successive PDI procedures was monitored. Data of the MBCs after PDI at days 5 and 10 are presented in tab. 1. No significant variations in MBCs values were observed for the tested strains.

Table 1. Multistep selection of PDI-resistant microorganisms with Zn-TBut4PyP

Strain	Initial MBC for Zn-TBut4PyP (μg/ml)	Resistance selection	
		Day	MBC (μg/ml)
S. aureus 62-A	0,5	5	0.5
		10	0.5
E. coli K-12	1	5	1
		10	1

Data are results of multistep resistance selection studies using Zn-TBut4PyP as photosensitizer in the presence of light activation and repeated MBC determination after days 5 and 10.

Many traditional antimicrobial agents act by inhibiting metabolic processes and therefore they need to be introduced and accumulated inside the cell to exert their action within the cytoplasm. This process often requires transport mechanisms, such as porins, which allow the influx of low molecular weight compounds (<700 Da) and exclude compounds above this molecular weight [19]. For PDI cationic drugs with high molecular weight, such as Zn-TBut4PyP (>1,000 Da), it is not necessary to penetrate the microbial cell [5, 17]. Specific adhesion of PS to these structures is usually considered enough for light-activated destruction of the target cells. Thus, target cells do not have a chance to develop resistance by stopping uptake, increasing metabolic detoxification or increasing active release (efflux) of the drugs [21]. The presence of multiple positive charges enables the PS agent to interact with the negatively charged outer cell wall areas of bacteria, in particular with the negatively charged lipopolysaccharides of Gram-negative bacteria, increasing the efficiency of the photoinactivation processes [1].

In addition, none of the capabilities of resistant microorganisms listed above protects against oxidative destruction of cell wall by singlet oxygen. Antioxidant enzymes such as peroxide dismutase, catalase and peroxidase give protection against some ROS, but not against the cytotoxic effect of singlet oxygen. Indeed, singlet oxygen has been shown to inactivate these enzymes [14]. At present it is generally accepted that the production of

singlet oxygen plays the key role in photosensitization, because a specific defense system against singlet oxygen itself is not present in bacteria [15]. Therefore, one of the most important features of the PS is to have a high $^1O_2$ quantum yield. In previous studies Gyulkhandanyan et al. showed that Zn-TBut4PyP had a rather high quantum yield of singlet oxygen (97%) and appeared as a promising PS for PDI of microorganisms [9].

Our experiments showed that the *S. aureus* 62-A and *E. coli* K-12 strains photosensitized with this PS (Zn-TBut4PyP), after 10 successive photodynamic procedures, did not develop resistance against the PDI. Despite the limited number of strains used in this experiment, we did not find a trend towards the development of resistance mechanisms after light activation with Zn-TBut4PyP. Few studies that tested the possible development of microbial resistance to PDI also proved that, microorganisms are not able to develop resistance [3, 20]. This suggests that PDI of microorganisms can be considered as a convenient innovative strategy for the treatment of localized infections.

In conclusion, we will say that further research is needed with a large number of microorganisms, as well as resistant strains in order to confirm these preliminary, but encouraging results.

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