

Comparative analysis of the photobactericidal action of selected tetrapyrrole compounds on Gram-positive and Gram-negative bacteria

A. Peèkaitytė^a,
R. Daugelavièius^a,
A. Sadauskaitė^a,
V. Kirvelienė^a,
R. Bonnett^b,
E. Bakienė^{a*}

^a Vilnius University, Department of Biochemistry and Biophysics, M. K. Èiurlionio 21, LT-03101 Vilnius, Lithuania

^b Department of Chemistry, Queen Mary, University of London, Mile end Road, London E1 4NS, UK

We have analysed the photobactericidal efficacy of four tetrapyrrole compounds on several strains of Gram-negative and Gram-positive bacteria. The bacterial cells were photosensitised with the following derivatives: uncharged meso-tetra(3-hydroxyphenyl)chlorin (mTHPC); cationic tetrakis (N-ethylpyridinium-4-yl) porphyrin tetratosylate (TN-Et-PyP); zinc phthalocyanine tetrasulfonate (ZnPcS₄) and aluminium phthalocyanine tetrasulfonate (AlPcS₄) (both anionic). The phototoxic activity of photosensitisers against all bacterial strains tested has been found to increase in the order AlPcS₄ < ZnPcS₄ < mTHPC < TN-Et-PyP. Gram-negative bacteria appeared to be more refractory to photodynamic action of all the tetrapyrrole derivatives tested. Gram-negative bacteria possessing a truncated lipopolysaccharide (LPS) chains were more sensitive to the phototoxic action of mTHPC than cells with wild-type LPS. Sensitivity of Gram-negative cells to photokilling by mTHPC could be enhanced by reducing the LPS content of bacterial cell envelope by Tris-EDTA treatment.

Key words: photodynamic antimicrobial chemotherapy (PACT), neutral, anionic and cationic photosensitisers, bacterial cell wall composition

INTRODUCTION

During the past decades the rapid growth of the medical industry with the widespread use of antimicrobials has changed the balance in the ecosystems of pathogenic microorganisms – more adapted bacterial strains, resistant to the most common bactericidal agents have emerged. The immediate development of alternative antiinfection modalities has become one of the highest priorities of medicine and biotechnology [1]. Photodynamic antimicrobial chemotherapy (PACT) presents a promising alternative to the use of antibiotics and antiseptics to combat resistant bacteria for therapeutic purposes, particularly for the treatment of localized infections [2]. PACT relies on a photosensitizing compound, light and oxygen in order to generate toxic products which result in the eradication of target cells. The photosensitiser, when exposed to light of an appropriate wavelength, is excited to a higher energy state; energy transfer to oxygen and to other surrounding substances produces reactive species such as singlet oxygen and free radicals, which act on various cons-

tituents of the bacterial cells resulting in cell death [2]. The multiplicity of targets makes it difficult for the cells to develop resistance, and this is one of the advantages of PACT [2]. Lethal photosensitisation of microbial cells was first demonstrated more than 100 years ago [3, 4]. However, since the discovery and systemic use of antibiotics, the photodynamic approach has not been employed in antibacterial therapy until recent years. It may be noted that the analogous photosensitisation-based technique named photodynamic therapy (PDT) has been successfully exploited within the past several decades for the treatment of cancer [5, 6]. Currently, PACT is gaining increased acceptance; several groups are investigating antibacterial, antiviral and antifungal properties of photodynamic treatment [2, 7–10]. One of the present directions in PACT development is a search for new photosensitisers with improved photobactericidal properties. There is a considerable current interest in the application of new generation photosensitisers based on various molecules containing a tetrapyrrole unit [11, 12]. Photosensitisation with different tetrapyrrole compounds has been shown to be efficient for the killing of cancerous and bacterial cells [7, 11].

*Autor for correspondence. E-mail: elena.bakienė@gf.vu.lt

The first aim of this study was to assess the photodynamic antibacterial properties of some tetrapyrrole compounds bearing different charges: uncharged meso-tetra(3-hydroxyphenyl)chlorin (mTHPC); cationic tetrakis(N-ethylpyridinium-4-yl)porphyrin tetrasulfate (TN-Et-PyP); zinc phthalocyanine tetrasulfate (ZnPcS_4) and aluminium phthalocyanine tetrasulfate (AlPcS_4) (both anionic). Apart from this physicochemical criterion, mTHPC, ZnPcS_4 and AlPcS_4 were chosen for this investigation as second-generation sensitizers with favourable spectroscopic and photochemical properties that have received considerable interest for use in PDT [5, 13, 14]. They are devoid of local and systemic toxicity and their photosensitizing properties have been evaluated extensively *in vitro* and *in vivo* [14–16]. Al and Zn phthalocyanines have been shown to be very effective for blood sterilization [15, 17]. To our knowledge, no data currently exist regarding the effect of mTHPC and AlPcS_4 mediated photosensitization on bacterial cells. ZnPcS_4 photobactericidal properties were investigated by some groups, but controversial data were obtained [18]. There appear to have been no previous published reports of the ability of TN-Et-PyP to act as a photosensitizing agent.

The second goal of this work was to evaluate the dependence of susceptibility of bacteria to photodynamic action on the structure of the bacterial cell wall. For this purpose, certain Gram-negative (*Escherichia coli* and *Salmonella enterica*) and Gram-positive (*Bacillus subtilis* and *Bacillus thuringiensis*) species were examined as the representatives of the best characterized bacteria commonly used in laboratory investigations. Besides, epidemiologically and ecologically relevant pathogenic counterparts of the first two species are ubiquitous throughout the world and can be regarded as targets for eradication.

The third goal of this investigation was to use the disorganization of the protective barrier of the bacterial envelope by permeabilizing agents to enhance the efficiency of photobactericidal action.

MATERIALS AND METHODS

Reagents. mTHPC and ZnPcS_4 were prepared as described in the literature [19, 20]. TN-Et-PyP was prepared by treating tetra(pyridin-4-yl)porphyrin with ethyl *p*-tosylate in dimethylformamide using the general procedure of Pasternack and coworkers [21]. mTHPC was dissolved in ethanol, TN-Et-PyP and ZnPcS_4 were dissolved in water as 1 mg/ml stock solutions. Aluminium phthalocyanine tetrasulfate (AlPcS_4) was purchased from Porphyrin Products, USA. The stock solution was prepared in Dulbecco's phosphate buffered saline (DPBS) (5 mg/ml). All stock solutions were stored at -20°C in the dark. All experiments were performed using dilu-

tion of the stock solutions with cell incubation media. EDTA and Tris were from Sigma. Other reagents were of analytical grade.

Target bacteria. *Escherichia coli* K12 strain AN180 (F⁻, arg, thi, xyl, str-r) was kindly provided by Prof. F. Gibson (National University of Australia). *Salmonella enterica* serovar Typhimurium strains DS88 (SL5676 Sm^r pLM2) and SL1102 (rfaE^b) were kindly provided by Prof. D. H. Bamford (University of Helsinki, Finland). *Bacillus subtilis* strain WB746 (wt) was from the collection of the Institute of Biochemistry, Vilnius, Lithuania. *Bacillus thuringiensis* (wt) was the generous gift of B. Sharga (Uzhgorod State University, Ukraine). The cells were grown in Luria–Bertani medium at 37°C with aeration to mid-log phase, harvested by centrifugation (5000 g, 10 min) and resuspended in a small volume of 0.1 M potassium phosphate buffer (pH 7.5). The cell suspension was kept on ice until used. Bacterial cell outer membrane labilization was achieved by 100 mM Tris/HCl-10 mM EDTA treatment at 37°C for 10 min.

Photodynamic treatment. Cell exposure to photosensitizers and light was carried out in flat-bottomed 96-well plates. 100 μl of bacterial suspension in 0.1 M potassium phosphate buffer (pH 7.5) at an approximate density of 1×10^5 cfu/ml and an appropriate concentration of the photosensitizer were added to each well. After incubation for appropriate times at 37°C in the dark, bacterial suspensions, both with and without photosensitizers, were exposed to light, as follows: cells treated with ZnPcS_4 , AlPcS_4 or mTHPC were exposed to light from LED array UNIMELA-660 ($\lambda = 660$ nm) and cells treated with TN-Et-PyP from LED array UNIMELA-509 ($\lambda = 509$ nm), the fluence rate being 10.5 W/m² and 21 W/m², respectively (VU Laser Research Centre, Lithuania).

Estimation of the viability of bacterial cells. Bacterial cell viability was defined as cell capability to form colonies on a solid nutrient gel. After photosensitizer and/or light exposure, the bacterial cells were serially diluted in 0.1 M potassium phosphate buffer (pH 7.5) and duplicate 50 μl aliquots were spread over the surfaces of LB nutrient broth agar plates. The colonies appearing on the plates were counted after overnight incubation at 37°C in the dark.

Data analysis. The data are presented as means \pm standard error (SE) from 2–5 independent experiments. SigmaPlot 2001 for Windows version 7.101 software was used for the statistical analysis.

RESULTS AND DISCUSSION

The photosensitized inactivation of microorganisms is a complex phenomenon and depends on many parameters. In the present study, we consider the

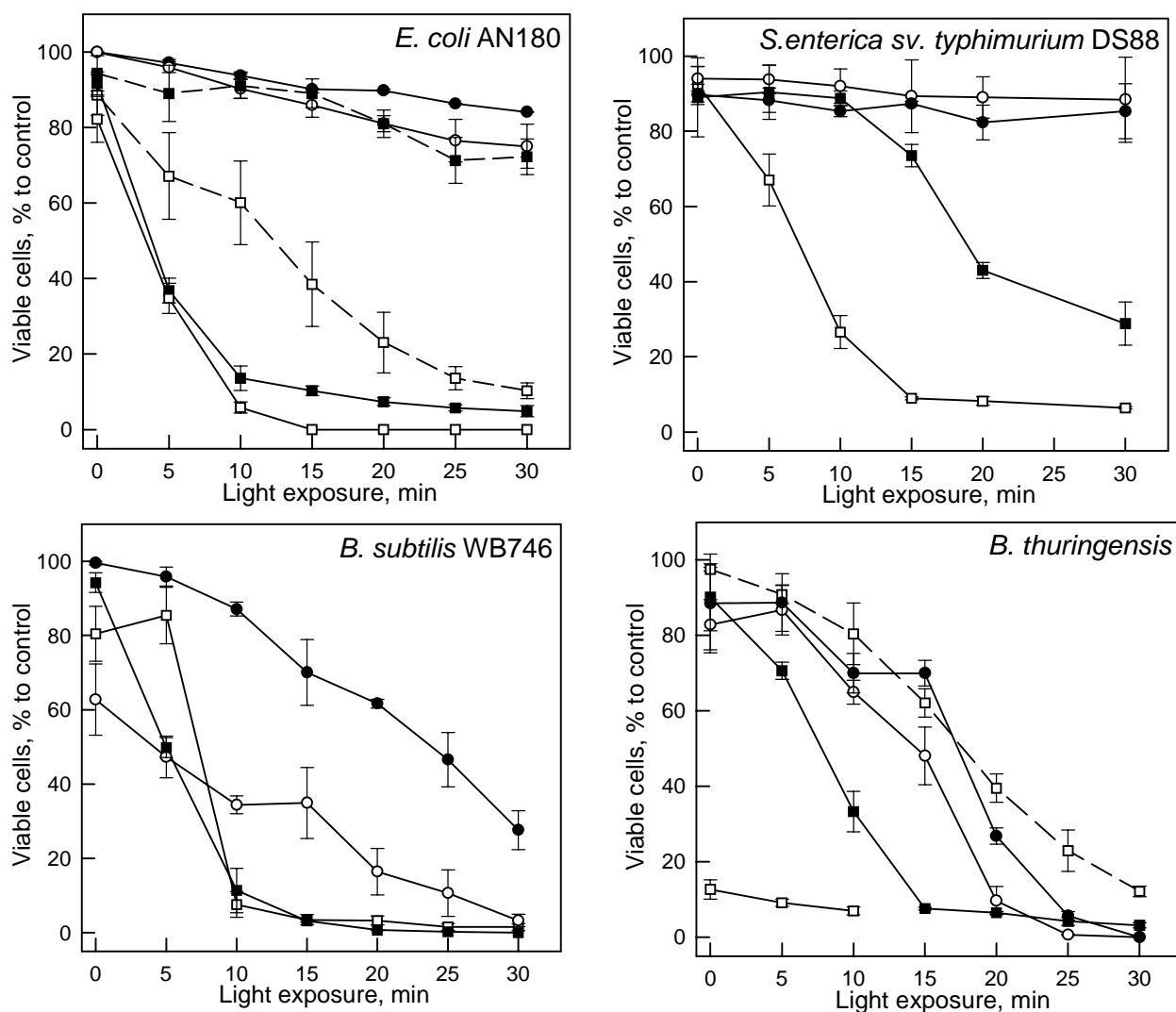


Fig. 1. Photobactericidal action of mTHPC, TN-Et-PyP, ZnPcS₄ and AlPcS₄ on Gram-negative (*E. coli* AN180 and *S. enterica* sv. Typhimurium DS88) and Gram-positive (*B. subtilis* WB746 and *B. thuringiensis*) bacterial cells

All the cells were incubated in 0.1 M potassium phosphate buffer (pH 7.5) at 37 °C in the dark for the following times: *E. coli* AN180 cells with mTHPC, overnight; with the other three dyes, for 30 min; all other bacterial strains with all photosensitisers, for 30 min. Then the cells were exposed to light at $\lambda = 509$ nm (TN-Et-PyP) or 660 nm (mTHPC, ZnPcS₄ and AlPcS₄) for the times indicated, diluted and plated.

Symbols: open circles, ZnPcS₄; closed circles, AlPcS₄; open squares, TN-Et-PyP; closed squares, mTHPC

Concentrations of photosensitisers: *E. coli* AN180, ZnPcS₄, 60 µg/ml; AlPcS₄, 60 µg/ml, TN-Et-PyP, 5 µg/ml (solid line) and 1 µg/ml (dashed line); mTHPC, 5 µg/ml (solid line) and 1 µg/ml (dashed line); *S. enterica* sv. Typhimurium DS88, ZnPcS₄, 60 µg/ml; AlPcS₄, 60 µg/ml; TN-Et-PyP, 2.5 µg/ml; mTHPC, 2.5 µg/ml; *B. subtilis* WB746, ZnPcS₄, 30 µg/ml; AlPcS₄, 30 µg/ml; TN-Et-PyP, 0.1 µg/ml; mTHPC, 0.5 µg/ml; *B. thuringiensis*, ZnPcS₄, 30 µg/ml; AlPcS₄, 30 µg/ml, TN-Et-PyP, 0.5 µg/ml (solid line) and 0.1 µg/ml (dashed line); mTHPC, 0.5 µg/ml

effect of the charge on the photosensitiser, dye concentration, the incubation time previous to light exposure and the illumination time on the irreversible destruction of bacteria with different cell envelope structures. Relevant data concerning the photobactericidal efficacy of four different tetrapyrrole compounds (uncharged mTHPC; cationic TN-Et-PyP; and anionic ZnPcS₄ and AlPcS₄) against both Gram-negative (*E. coli* and *S. enterica* sv. Typhimurium) and Gram-positive (*B. subtilis* and *B. thuringiensis*) bacteria are given in Fig. 1. One can see that bacteria of all the strains tested were eradicated to

some extent by irradiation in the presence of mTHPC and TN-Et-PyP; however, the degree of photodamage was dependent upon the dye as well as the type of bacterium. Figure 1 demonstrates that the cationic derivative was generally more photoactive than the uncharged photosensitiser: a comparable or even greater drop in cell viability was achieved at TN-Et-PyP concentrations less than that of mTHPC. ZnPcS₄ and AlPcS₄ showed rather weak photobactericidal properties: they were active against Gram-positive bacteria only at concentrations 60–300-fold higher than those of mTHPC and TN-Et-

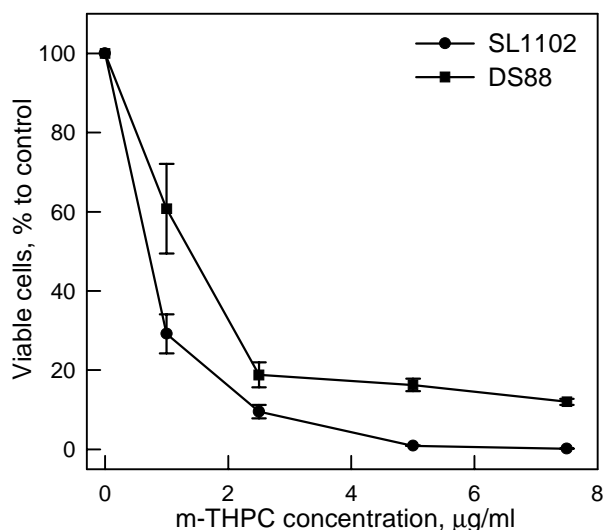


Fig. 2. Phototoxic action of mTHPC on two strains of *S. enterica* sv. Typhimurium, differing in their OM LPS length. Cells of *S. enterica* sv. Typhimurium strain DS88 (wt) or SL1102 (deep rough LPS mutant) were incubated with mTHPC in the dark for 60 min, exposed to light at $\lambda = 660$ nm for 30 min, then diluted and plated

PyP, respectively, and under our experimental conditions did not show any photosensitizing activity against Gram-negative bacteria. Gram-negative microorganisms appeared to be much more refractory to the photodynamic action of all the four dyes tested, compared with Gram-positive ones. The increase of the illumination time while keeping the concentrations of the dyes constant resulted in a greater destruction of bacteria. Irradiation of the bacteria in the absence of photosensitisers had no detectable effect on bacterial viability (data not shown), and the test compounds themselves, except ZnPcS_4 with *B. subtilis* and TN-Et-PyP with *B. thuringiensis*, did not exert significant dark toxicity at the concentrations tested (Fig. 1).

Besides dye concentration and illumination time, the photobactericidal effect of a given photosensitiser was dependent on the time of dark incubation of the bacteria with dye before the illumination. Data of our experiments showed that mTHPC failed to photosensitise *E. coli* cells after 3 h of dark incubation; however, it exhibited strong phototoxic properties against this type of bacteria following overnight dark incubation (compare Fig. 1 and Fig. 3). Much less pre-illumination incubation time (0.5–1 h) was sufficient for photokilling the other bacterial strains with mTHPC, as well as all the bacteria tested, with TN-Et-PyP. Gram-negative bacteria remained resistant to the photodynamic action of phthalocyanines following the dark pre-incubation ranging from 0.5 to 18 h (unpublished results, see also Fig. 1). The differences of pre-illumination times required to manifest the photokilling efficiency of photosensitisers are probably related to the duration of penetration of the dye through the cellular diffu-

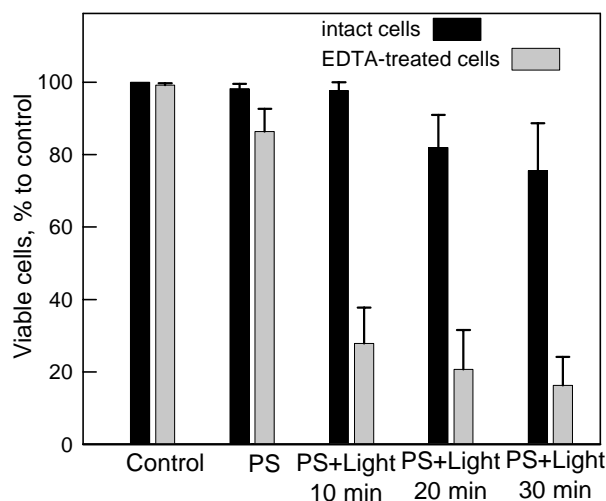


Fig. 3. Photodynamic effect of mTHPC on the viability of intact and EDTA-treated *E. coli* AN180 cells. Bacterial cells were pretreated with EDTA as described in Materials and Methods. The cells were incubated with 2.5 $\mu\text{g/ml}$ of mTHPC in the dark for 3 h, exposed to light at $\lambda = 660$ nm for the times indicated, then diluted and plated. PS, cells incubated with photosensitiser without light exposure

sion barriers to the appropriate targets for photochemical attack within the bacterial cell and demonstrate the importance of this stage in the total cell photodamage process. A significant contributing factor can be the intracellular localization and binding site of a photosensitiser, which is highly affected by the structure and charge of the dye.

The influence of the net charges of the photosensitisers, e.g., porphyrins, on the spectrum of their photobactericidal activity has been investigated in other laboratories, and it has been shown that cationic derivatives are generally more photoactive than anionic or neutral ones, especially against Gram-negative bacteria [2, 22, 23]. The results reported here are consistent with these observations, since photobactericidal efficacy of the sensitizers tested in this study was found to increase in the following sequence: $\text{AlPcS}_4 < \text{ZnPcS}_4 < \text{mTHPC} < \text{TN-Et-PyP}$, the cationic dye being the most potent, while the anionic ones were rather weak photobactericides (Fig. 1). Several reports indicate that the failure of photodynamic treatment to kill the bacteria can be due to a lack of cellular uptake of exogenous photosensitisers [24, 25]. Indeed, the uptake of the anionic dyes by bacteria is impeded by the repulsion forces between the dye and the negatively charged cell surface. However, the issue appears to be more complex than simply the levels of gross cellular uptake, since some anionic porphyrins and other dyes appeared to be taken up by Gram-negative bacteria, but not to induce photosensitization, probably because these photosensitisers localize in non-vital regions of the target cells [23]. On the other hand, photobactericidal activity of extracellu-

larly localized (*e.g.*, polymer immobilized) dyes was demonstrated repeatedly [2, 7, 26], provided they possessed a sufficient singlet oxygen-generating efficiency. In those cases the sites of photodynamic damage are presumably limited to the cell surface due to the very short diffusion distance of $^1\text{O}_2$, the most important mediator in the photobactericidal activity [2, 7]. Hence, the precise mode of photobactericidal action is to be found at more refined levels, *e.g.*, the details of the interaction of the given photosensitiser structure with the specific microenvironment.

Our findings concerning the different susceptibility of Gram-negative and Gram-positive bacteria to photodynamic action match the previously reported data of other groups [2]. This distinction can be attributed to the structural and compositional differences between the cellular envelopes of Gram-negative and Gram-positive organisms, in particular, to the existence of the outer membrane (OM) in Gram-negative bacteria. OM is an asymmetric bilayer [27]: the inner layer consists of phospholipids and the outer layer of lipopolysaccharide (LPS). In our case, LPS acts as a permeability barrier preventing the access of the photosensitiser molecules to the underlying sites, as well as intercepting the cytotoxic reactive species (*e.g.*, singlet oxygen), which are generated by photosensitisers. Most common Gram-positive bacteria lack a barrier comparable to the OM of Gram-negative bacteria, and for this reason demonstrate the higher sensitivity to the combined action of light and dye [2].

Our investigations concerning the protective role of bacterial LPS are presented in Figs. 2 and 3. It can be seen (Fig. 2) that *S. enterica* *sv.* Typhimurium strain SL1102 (heptoseless deep rough LPS mutant) possessing a truncated LPS chain is more sensitive to the phototoxic action of mTHPC than *S. enterica* *sv.* Typhimurium DS88 cells with wild-type LPS. It has been shown previously that chelating agents such as ethylenediaminetetraacetic acid (EDTA) effect the release of up to 50% of the LPS from the outer membrane of Gram-negative cells [27]. The results of our experiments showed (Fig. 3) that EDTA-treated *E. coli* cells were more prone to lethal photosensitisation with mTHPC than native cells of the same strain, probably because of the reduced LPS content in EDTA-modified bacterial cells. Similar results were obtained in other laboratories with some other photosensitisers and different strains of Gram-negative bacteria [2, 28]. From the practical point of view, these findings make it possible to enhance the susceptibility of Gram-negative bacteria to photodynamic treatment and to broaden the field of PACT application.

However, our investigations of the relative effectiveness of mTHPC against two types of Gram-negative bacteria revealed (Figs. 1 and 2) that *E. coli* AN180 (as well as some other *E. coli* strains – da-

ta not shown) was more resistant to the photodynamic action of this photosensitiser compared to *S. enterica* *sv.* Typhimurium DS88. This was unexpected, since according to the literature [27, 29] *E. coli* has a similar or less extensive LPS of OM than *S. enterica* *sv.* Typhimurium, so *E. coli* should be equally or more sensitive to the action of illuminated dye [29]. These discrepancies indicate that apart from the Gram-negative character other factors can influence the susceptibility of bacterial cells to photodynamic action. With regard to mTHPC, we hypothesize that the following factors can contribute to the enhanced tolerance of *E. coli* cells to the phototoxic action of this dye: (i) some unidentified OM structure peculiarities of the strain tested; (ii) expression of some antioxidant enzymes such as superoxide dismutase, which were shown to be present in some bacterial strains to deal with environmentally occurring oxygen radicals [30]; (iii) the efflux system extruding mTHPC out of the bacterial cells in an energy-dependent manner. The efflux systems of bacterial cells which expel a wide range of compounds, including drugs, were discovered in many bacterial species and are responsible for drug resistance phenomena [31]. Though there has not been any report about the operation of similar efflux systems for photosensitisers, the possibility of their existence cannot be ruled out.

Further work is now in prospect for the study of the cellular and molecular mechanisms underlying the photobactericidal action of mTHPC and TN-Et-PyP as these dyes appear to show considerable promise for PACT.

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**A. Pečkaitytė, R. Daugelavičius, A. Sadauskaitė,
V. Kirvelienė, R. Bonnett, E. Bakienė**

PASIRINKTŲ TETRAPIROLO DARINIŲ FOTOKBakteriCIDINIO POVEIKIO GRAM-TEIGIAMOMS IR GRAM-NEIGIAMOMS BAKTERIJOMS PalyginamoJI ANALIZĖ

Santrauka

Ūiame darbe tyrėme kelio fotosensibilizatorių – tetrapirolo darinio, turinio skirtinga krūvā katijonio tetrakio (N-etilpiridin-4-il) porfirino tetratosilato (TN-Et-PyP); dviejū nei-giamai ākrautū daŗū – cinko ir aliuminio ftalocianinū tetra-sulfoninės rūgūties tetranatrio druskū (ZnPcS₄ bei AlPcS₄, atitinkamai) bei neutralaus mezotetra(m-hidroksifenil)chlo-rino (m-THPC) – fototoksinā poveikā gram-neigiamoms bei gram-teigiamoms bakterijoms. Nustatyta, kad tirtūjū daŗū fototoksiokumas didėja AlPcS₄ < ZnPcS₄ < mTHPC < TN-Et-PyP eilėje. Visū tirtū tetrapirolo darinio fototoksinis po-veikis gram-teigiamoms bakterijoms buvo stipresnis negu gram-neigiamoms, turinio sudėtingesnės struktūros ap-vaikalėlā Ūio bakterijū jautrumas fototoksiniam m- THPC poveikiui padidėdavo paŗeidus lipopolisacharidinā (LPS) lās-telio Ūorinės membranos sluoksnā Tris-EDTA miūiniui.