

Molecular Determinants of Porphyrin Photosensitizer-Mediated Bacterial Photoinactivation

Fatima Sequeira

Kuwait University

William Knight

Barrow Neurological Institute

Artak Tovmasyan

Barrow Neurological Institute

Ludmil Benov

`ludmil.benov@ku.edu.kw`

Kuwait University

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Abstract

The widespread and increasing resistance of bacteria to antibiotics creates a demand for development of alternative methods for microbial eradication. Photodynamic inactivation (PDI) is a promising approach because of its demonstrated efficacy against a wide range of drug-resistant microorganisms. The practical application of PDI needs photosensitizers (PSs) with high quantum yield, low toxicity against the host, and potent antimicrobial activity. Design of such PSs requires a detailed understanding of how molecular structure influences PDI efficacy. This work investigated the PDI activity of a series of *meso*-substituted *N*-ethylpyridinium-2-yl and pentafluorophenyl substituted porphyrins bearing zero to four positive charges. Results demonstrated that the highest bactericidal activity against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) microorganisms was displayed by the asymmetric dicationic porphyrin with adjacent positive charges (*cis*-H₂DiE). Despite having a similar molecular structure, comparable singlet oxygen quantum yield, and similar lipophilicity, its symmetric regioisomer with two opposite positive charges (*trans*-H₂DiE) was substantially less photoeffective. Pronounced differences were also observed in the PDI sensitivity of Gram-negative and Gram-positive bacteria. Investigations with membrane-impermeable fluorescent agents revealed that membrane permeabilization was the major cause for photo-inactivation of microbes. The combination of moderate lipophilicity and adjacent positive charges facilitates deeper penetration of the PS in the membrane lipid bilayer, positioning the PS in an environment with higher oxygen availability and closer proximity to unsaturated fatty acids, thereby favoring oxidative membrane damage. These findings demonstrate that in addition to lipophilicity and charge number, the molecule symmetry and spatial distribution of charges (*ortho* vs *meta* vs *para* and adjacent vs opposite), the tridimensional shape of the molecule, its flexibility and bulkiness, should be considered when designing PSs for potential clinical applications.

Key Points

Position of cationic substituents predicts photosensitizers localization and efficiency

Photosensitizer localization determines its bactericidal photoactivity

Adjacent positive charges at position and moderate lipophilicity are necessary for high antimicrobial activity

Introduction

In recent years, eradication of pathogens by a combination of light and various types of dyes, including porphyrins and phthalocyanines, termed Photodynamic Inactivation (PDI) or antimicrobial Photodynamic Therapy (aPDT), has attracted attention due to its success in fighting a wide range of organisms, including multidrug-resistant bacterial strains, viruses, fungi and protozoa [1–7]. In PDI, a non-toxic, light-absorbing compound called a photosensitizer (PS) becomes excited by illumination with visible light, triggering type I and type II photoreactions, which generate radicals and singlet oxygen [8]. The photo-

generated reactive species attack and damage critical cellular components, killing unwanted cells. Because the life of such reactive species is very short in biological environment, damage is limited to a very small area surrounding the PS molecule. This makes uptake and subcellular distribution of the PS a key factor determining photodynamic efficacy and at the same time contributes to treatment selectivity [9].

Irrespective of its advantages as a highly selective treatment, PDI is limited by the lack of highly efficient PSs with low dark toxicity. Systemic studies investigating how molecular structure affects PDI efficacy are scarce, which hampers attempts for rational design of PS with desired photodynamic properties.

Our previous studies using cationic *ortho* Zn(II) *N*-alkylpyridyl-porphyrin-based PSs (ZnPs) demonstrated that increasing the length of the aliphatic chains at all four *meso* positions from 1-C (methyl) to 6-C (hexyl) produced an amphiphilic, powerful *ortho*-hexyl PS, which efficiently photo-inactivated bacteria and yeasts [5–7, 10].

While the effects of PSs lipophilicity on PDI have been relatively well explored, considerably less is known about how the number, position, and spatial distribution of positive charges influence the antimicrobial photodynamic activity [11–15]. In all listed publications, *meso*-substituted compounds bearing positively charged pyridyl groups have predominantly charge localization at *para* position. Previous works have demonstrated that shifting the positively charged pyridyl nitrogen from *para* to *ortho* position in *meso*-substituted pyridyl porphyrins had a profound effect on their interactions with cellular components and affected their biological activity [16–18].

The aim of this study was to investigate how the number and spatial distribution of positive charges influence the photoinactivation of bacteria by a series of porphyrins. Specifically, we examined the photodynamic activity of *ortho* *N*-ethylpyridyl tetrapyrroles containing zero to four positive charges against Gram negative (*E. coli*) and Gram positive (*S. aureus*) bacterial species. By comparing structurally related compounds with different numbers and arrangements of cationic substituents, including asymmetric and symmetric dicationic porphyrins, we demonstrate structure–activity relationships governing antimicrobial PDI and identify molecular features associated with enhanced bactericidal activity.

Materials and Methods

Strains and Growth Conditions

The strains used in this study were Gram-negative, *Escherichia coli* GC4468 [19] and Gram-positive, *Staphylococcus aureus* ATCC25923 [20].

E. coli cultures were grown overnight in Luria Bertani (LB) medium and *S. aureus*, in Brain Heart Infusion (BHI) broth (BD Biosciences, USA), in a shaking water bath at 200 rpm and 37°C. For preparation of LB plates, 15 g of agar were added to 1 L of liquid medium.

To avoid photogeneration of toxic media components, for all PDT experiments the cells were washed and resuspended in PBS containing 0.2% glucose (PBS-glucose). Except where otherwise indicated, experiments were performed in 96-well plates. Portions, (100 μ l/well) of cell suspensions with OD_{600nm} = 0.5 were transferred into triplicate wells and the tested compounds were added to final concentrations indicated in the figure legends. The plates were illuminated for 15 minutes at 64 mW/cm² by an incandescent broad spectrum light source whose spectral characteristics have been previously published [5].

Effect of PDI on cell metabolic activity was assessed by the MTT assay [21] and cell viability was determined by CFU (colony forming unit). For CFU determination, after illumination, the samples were diluted and spread evenly on LB agar plates. Plates were incubated for 24/48 h at 37°C in the dark and colonies were counted. In all experiments, dark and illuminated controls were run in parallel.

Photosensitizers

The structures of the tested PSs are shown in **Fig. 1**. Their synthesis and characterization have been previously described [22].

Cellular uptake of the photosensitizers

Binding of the tested compounds to bacterial cells was assessed as described by Simoes et al. [14]. A bacterial suspension in PBS-glucose ($\sim 5 \times 10^8$ cells/ml) was incubated at 37°C and 200 rpm with 5 μ M of the tested compounds. One ml aliquots were taken at 15, 30 60, 120 and 240 minutes, rapidly centrifuged at 4°C, and washed with ice-cold PBS. The washed pellet was resuspended in 2% SDS/0.1 M NaOH [23]. The samples were incubated at room temperature in the dark for 24 hours or until the solution became clear. The concentration of each compound was determined by recording the fluorescence emission spectra and calculating the areas under the peaks. Excitation wavelength corresponding to the Soret band of each compound was used. Since fluorescence could be affected by cellular fluorophores, a spectrophotometric assay was performed in parallel by recording the absorption spectra and calculating the area under the Soret peak. The amount of cell-bound PSs was calculated using a separate standard curve for each PS, obtained by dissolving pure compound in 2% SDS/0.1 M NaOH.

Propidium iodide staining

Immediately after the illumination, propidium iodide was added to a final concentration of 15 μ M and samples were incubated 10 minutes in the dark. Each sample was diluted to a final volume of 3.0 ml and fluorescence intensity was measured at λ_{ex} 535 nm and λ_{em} 615-620 nm on a Shimadzu RF-6000 spectrofluorometer.

Singlet Oxygen Sensor Green (SOSG) staining

E. coli cells were washed with PBS and resuspended in PBS-glucose to $OD_{600nm} \sim 1.2 - 1.3$. SOSG stock solution (500 μM) and work solution (final concentration in the samples 2 μM) were prepared in deoxygenated methanol immediately before use. SOSG was added to each sample and images were obtained 5 – 10 minutes after the addition of SOSG on a confocal fluorescent microscope Carl Zeiss, model LSM 980. Fluorescence emission intensity was determined using software Zen 3.3 Blue Edition.

Data analysis

Experiments were repeated at least two times, each sample run in triplicate. One Way Analysis of Variance (ANOVA) was performed using SigmaPlot version 11.0 and p value < 0.05 was accepted as statistically significant. Data is presented as mean $\pm$ SD.

Results

Among the main requirements for an efficient PS is high singlet oxygen quantum yield [24]. Our previous investigations demonstrated that the tested compounds can be arranged in the following order of decreasing $^1O_2$ quantum yield ***trans*-H₂DiE** $\geq$ ***cis*-H₂DiE** > **H₂Tri** $\geq$ **H₂TE** > **H₂ME** > **H₂TF₅PhP** [22]. Since singlet oxygen and reactive species generated by type I photochemical reactions have short life and very short diffusion distances in biological environment [25, 26], another key factor determining PDI outcome is binding to and uptake of the PS by the target cells [27]. Among the parameters affecting cellular uptake of PSs are lipophilicity, and character, number and position of charges [5]. Lipophilicity of the tested compounds progressively increased with the increase of the number of the fluorinated phenyl groups, following the order **H₂TF₅PhP** > **H₂ME** > ***trans*-H₂DiE** = ***cis*-H₂DiE** > **H₂Tri** > **H₂TE** [22].

The uptake of the PSs by *E. coli* did not correlate with compounds lipophilicity. H₂ME accumulated to the highest level in the cells, followed by *trans*-H₂DiE, *cis*-H₂DiE, and H₂TF₅PhP (**Fig. 2**). The uptake of the most hydrophilic compounds **H₂TE** and **H₂Tri** was negligible. **Fig. 2** also shows that PSs were rapidly taken up, reaching maximal cellular concentration within the first 30 – 60 minutes of incubation.

Exception was the amphiphilic ***cis*-H₂DiE**, whose uptake continued to increase during the entire length of incubation. Slow, gradual increase, but at much lower level, was observed for the tricationic compound **H₂Tri**. In contrast, the amount of cell-bound PSs ***trans*-H₂DiE**, **H₂ME**, **H₂TF₅PhP** decreased with the time of incubation, reaching almost half of its 60' concentration within 4 hours. A plausible explanation about such decline is decomposition of the compounds. Based on these results, a period of 60 minutes incubation of cells with the PSs was selected for the PDI experiments.

The next question to be answered was, does cellular uptake determine photodynamic antibacterial activity. Initially we tried to test the PDI activity of the PSs with a simple and fast screening assay. The MTT colorimetric test is commonly used as a surrogate assay for cell viability [28]. Since it is based on

enzymatic, NAD(P)H-dependent reduction of MTT to purple formazan, in reality it is a measure of cells metabolic activity [29].

Fig. 3A shows that at 5 μ M, only the *cis* isomer (***cis*-H₂DiE**) completely suppressed MTT reduction. About 60% suppression of formazan production was observed by the *trans* isomer, followed by the tricationic compound **H₂Tri** (~ 25% suppression of MTT reduction). The rest of the tested compounds did not demonstrate significant photodynamic inhibition of *E. coli* metabolism. None of the tested PSs affected MTT reduction in the absence of light.

Irrespective of its common use to determine “viability”, the MTT assay, which is redox reaction-based, is entirely dependent on activity of enzymes and availability of NAD(P)H, and as result, is prone to artifacts [30-34]. The colony forming unit (CFU) assay has been accepted as the gold standard for assessment of cell viability [35-39].

Fig. 3B demonstrates that when plating and enumeration of colonies was performed after the illumination, the most efficient PS was again the compound with two adjacent positive charges (***cis*-H₂DiE**), followed by the tricationic PS (**H₂Tri**) and the dicationic isomer with two positive charges in opposite position (***trans*-H₂DiE**). The most hydrophilic (**H₂TE**) and the most lipophilic compounds (**H₂ME** and **H₂TF₅PhP**) displayed low PDT efficacy, not exceeding 40%. Based on these results, the tested compounds can be arranged in the following order of decreasing PDI activity: ***cis*-H₂DiE > H₂Tri > *trans*-H₂DiE > H₂ME > H₂TE $\geq$ H₂TF₅PhP**.

It is important to note that none of the two compounds that demonstrated the highest cellular uptake, PSs ***trans*-H₂DiE** and **H₂ME**, was among the top PSs with the highest PDI efficacy. Furthermore, the tricationic PS **H₂Tri**, which displayed the second lowest uptake came the second highest in PDI activity. These results, however, must be interpreted with caution, because the absence of growth is not a definite indicator of cell death [40]. Sublethal cell damage can induce the so called viable but non-culturable state [41, 42]. A universally accepted difference between the dead and viable cells is the intactness of the plasma membrane. Damage of the plasma membrane and loss of its barrier function is accepted as a clear indication of cell death [41].

A widely used method to determine plasma membrane damage is measuring the movement of normally non-permeable molecules across the membrane. Propidium iodide (PI) is often used for bacterial viability staining because it can cross only damaged membranes and serves as an indicator of membrane integrity. Inside the cell, PI binds to nucleic acids and emits red fluorescence. Results (**Fig. 3C**) show that the strongest fluorescent signal was detected when *E. coli* cells were subjected to illumination in the presence of the dicationic PS ***cis*-H₂DiE** with adjacent positive charges, followed by the tricationic compound **H₂TriE**, and the dicationic ***trans*-H₂DiE** with opposite positive charges. Thus, PI staining followed the order of PDI efficacy observed by colony counting. Since the cells were washed before the illumination, membrane damage was caused only by tightly bound PSs.

Photo-generated singlet oxygen is considered the main cell-damaging factor in photodynamic therapy [8]. Singlet oxygen sensor green (SOSG), a commercial cell-impermeable fluorescent probe, advertised to be specific for the detection of singlet oxygen, has been commonly used to prove $^1\text{O}_2$ production [43-47]. Results presented in **Fig. 4** show that the highest cellular SOSG fluorescence was emitted by cells illuminated in the presence of the dicationic amphiphilic PS **cis-H₂DiE**, followed by the tricationic **H₂Tri** and the dicationic PS **trans-H₂DiE**. Irrespective of the fact that the difference between **cis-H₂DiE** and **trans-H₂DiE** is only in the position of the positive charges, adjacent vs opposite, a big difference in SOSG fluorescence was observed. This cannot be explained by a difference in singlet oxygen quantum yields, because the difference between the two compounds with respect to Φ_D is very small (0.31 vs 0.33 [22]). Furthermore, our investigation revealed that fluorescent staining of cells with SOSG depends not on singlet oxygen but on plasma membrane permeability [48]. Only cells with compromised plasma membrane barrier function could be stained with SOSG and such staining could be achieved under anaerobic conditions and by treatments that do not involve $^1\text{O}_2$ [48]. No SOSG fluorescence was observed when *E. coli* was incubated with PSs in the dark. Thus, results presented in **Fig. 4** confirm that **cis-H₂DiE** is the most efficient in inflicting photo-dependent membrane damage.

Due to differences in their cell envelope, Gram-positive bacteria are more susceptible to photoinactivation than the Gram-negative organisms [49, 50].

Under the same experimental conditions, *S. aureus* appeared much more sensitive to photoinactivation by the tested PSs than *E. coli*. Even at concentration as low as 0.1 μM , all tested compounds except the most hydrophilic **H₂TE** and the most lipophilic **H₂TF₅PhP** inactivated more than 60% of the cells (**Fig. 5A**). The highest PDT efficacy, > 99%, was demonstrated by **cis-H₂DiE** followed by its *trans* analog. These two compounds, however, exerted a substantial dark toxicity, inactivating > 60% of *S. aureus* in the absence of light.

The dark toxicity of the *cis* and *trans* isomers at 0.1 μM suggests that for photoinactivation of *S. aureus*, these two PSs should be applied at much lower concentration. **Fig. 5B** shows that against *S. aureus*, the *cis* isomer was highly photoactive at a concentration as low as 0.02 μM (~ 94% of *S. aureus* inactivated) with negligible light-independent toxicity (< 20%). There was a progressive increase in the light-independent inactivation of *S. aureus* with the increase of the *cis* isomer concentration. Similar relationship was observed for the *trans* isomer but below 0.1 μM its photo-efficacy was much lower than that of its *cis* analog (**Fig. 5C**).

Gram-positive bacteria lack outer membrane and have quite porous peptidoglycan layer, which allow PSs to easily penetrate the plasma membrane and reach cellular interior [50, 51]. It is reasonable to expect, therefore, that the most efficient PSs would accumulate to the highest level in *S. aureus*. **Fig. 5D** shows that indeed **cis-H₂DiE** reached the highest concentration in *S. aureus*, followed by its *trans* analog. The uptake was faster than the uptake by *E. coli* and these two PSs reached maximal intracellular level within

the first 15 minutes of incubation. Among the rest of the tested PSs, only **H₂M** demonstrated measurable uptake by *S. aureus*, but much lower than the *cis* and *trans* isomers (**Fig. 5D**).

Discussion

We investigated the effect of number and spatial distribution of positive charges on photo-efficacy of porphyrin-based PSs against Gram-negative (*E. coli*) and Gram-positive (*S. aureus*) bacteria. A specifically designed series of porphyrins containing zero to four *ortho* *N*-ethylpyridinium and pentafluorophenyl moieties was used to systematically vary both the number and arrangement of positive charges. To determine the importance of cellular uptake of the PSs for PDT efficacy, the cells were thoroughly washed after incubation with the PSs and before illumination to remove unbound and loosely bound PSs. Under the selected experimental conditions, the most efficient PS against both *E. coli* and *S. aureus*, was the PS with two adjacent positive charges, ***cis*-H₂DiE**. Notably, despite having comparable singlet oxygen quantum yield and similar lipophilicity, the corresponding symmetric dicationic isomer with two opposite charges, ***trans*-H₂DiE**, was less effective. This finding indicates that overall charge number alone does not account for antimicrobial photodynamic activity and highlights the importance of the spatial arrangement of charges within the PS molecule. The enhanced activity of ***cis*-H₂DiE** is in agreement with previous reports demonstrating differences in the photodynamic activity of *cis* and *trans* porphyrin isomers in both prokaryotic and eukaryotic cells [11, 13, 14, 52, 53]. Engelmann and coworkers proposed that amphiphilic cationic compounds bearing adjacent positive charges can penetrate deeper in the membrane lipid bilayer than their isomeric counterparts [53]. Localization within the membrane places the PS in an environment with greater local oxygen availability and in close proximity to unsubstituted membrane lipids. Upon photoexcitation, the PS can therefore generate singlet oxygen and other reactive oxygen species near critical membrane components, promoting protein and lipid oxidation [8, 54]. Subsequent oxidative damage and free radical chain reactions compromise membrane integrity and barrier function, leading to cell death [55]. Our investigation demonstrated that *cis*-H₂DiE produced the highest plasma membrane permeabilization, coinciding with its highest PDT inactivation of *E. coli*. This finding is in line with the results reported by Lopes and coworkers, who reported that a photo-activated porphyrin-based PS bearing two adjacent positive charges was the most efficient in inducing membrane lipids peroxidation [13].

All other PSs studied caused substantially less membrane damage and were less efficient as antibacterials. The tested compounds can be arranged in the following order of decreasing bactericidal photoactivity: ***cis*-H₂DiE > H₂Tri > *trans*-H₂DiE > H₂ME > H₂TE > H₂TF₅PhP**.

Simoes and coworkers [14] investigated a series of similar porphyrins containing 1 to 4 positive charges and similarly demonstrated that antibacterial photoefficacy correlates with the number of charges and their spatial distribution. In their study, the highest PDT activity against *E. coli* was observed with a tricationic porphyrin, followed by the tetracationic compound, the *cis* dicationic PS with two adjacent charges, its *trans* isomer, and the monocationic PS. Similar trends were reported by Alves et al. [11].

Differences between those findings and the results of the present study may reflect important differences in experimental design. In the previous studies, bacteria were exposed to PSs for only 15 min before illumination, and photodynamic activity was subsequently assessed following prolonged (up to 4.5 hours) irradiation, at relatively low irradiance (4 mW/cm^2), with PSs remaining present in the medium [11, 14]. Under such conditions, a PS that is not internalized but remains loosely bound to cell surface, may damage the cell envelope, potentially facilitating subsequent penetration and photo-damage of sensitive cellular components [56, 57]. In addition, prolonged irradiation allows redistribution of the PSs within cells [58-60] and eventually may increase the possibility of PS photodegradation [61-63]. In contrast, our experimental design involved removal of unbound and loosely associated PS before illumination, thereby placing greater emphasis on the biological consequences of PS molecules that remain associated with the bacterial cells.

An additional consideration is the position of the pyridyl nitrogen relative to the porphyrin core. Unlike the PSs examined in several previous studies [11, 13, 14, 52, 64], which contained *para*-substituted pyridyl groups, the compounds investigated here contained *ortho*-substituted pyridyl groups. Positioning the positively charged pyridyl nitrogens at the *ortho* position restricts the rotation of the alkylpyridyl groups and favors an orientation approximately perpendicular to the tetrapyrrole plane [8]. This arrangement increases the effective steric bulkiness of the molecule, which precludes their interaction with nucleic acids. In contrast, the *para*-substituted pyridyl groups permit greater rotational freedom and allow the porphyrins to adopt a more planar conformation and can facilitate intercalation in nucleic acids [16-18]. Consistent with these structural differences, genomic DNA photodamage has been identified as a primary mechanism of bacterial photoinactivation by *para*-TMPyP [65], whereas *ortho*-substituted alkylpyridyl porphyrins have been reported to inactivate bacteria without detectable DNA damage [6]. Similarly, previous comparisons of methylpyridyl substitution at different positions have demonstrated that changing the substitution pattern (from *para* to *meta* position in TMPyP) can substantially alter biological activity [66]. These observations support the concept that the position of the cationic substituent is an important structural determinant of PS localization and photodynamic mechanism.

Our cellular uptake data further demonstrate that the total amount of PS associated with bacterial cells does not necessarily predict photodynamic efficacy. Simoes and coworkers [14] similarly reported that cellular binding of the *para*-substituted PSs did not directly correlate with photoactivity. In their study, the monocationic PS showed the highest uptake by *E. coli*, followed by the *trans* di-, tri- and tetracationic, and the *cis* dicationic compounds, despite the substantially different photodynamic activity of these compounds. In the present study, the uptake profile of the *ortho*-substituted compounds followed similar trend. The highest *E. coli* uptake was demonstrated by the monocationic **H₂ME**, followed by ***trans*-H₂DiE** > ***cis*-H₂DiE** > **H₂TF₅PhP** > **H₂Tri** ≥ **H₂TE**. Cellular binding of the tricationic **H₂Tri** and the tetracationic **H₂TE** was negligible. The apparently higher cellular uptake of the highly lipophilic, uncharged **H₂TF₅PhP**, should be interpreted with caution. Because of its high lipophilicity, this compound is prone to aggregation in aqueous environment, and aggregated material may be difficult to remove completely

during the washing. Consequently, the measured cellular association may overestimate the amount of monomeric PS that is genuinely associated with the bacterial cells.

The difference in the uptake observed for **cis-H₂DiE** between our study and the study [14] can be attributed to differences in the positions of the positive charges (*ortho* vs *para*) and the substituents attached to the pyridyl nitrogen (ethyl vs methyl). Our results confirm the conclusion of Simoes et al. [14] that uptake of the PSs by *E. coli* does not correlate with their antibacterial photo-efficacy. Such lack of correlation indicates that it is not the total amount of the compound that is bound to the cells but the localization of the PS that determines its bactericidal photoactivity.

The pattern of PS binding with *S. aureus* cells differed from that observed with *E. coli*. The highest uptake was demonstrated by **cis-H₂DiE**, followed by **trans-H₂DiE** and **H₂ME**. The tricationic, the tetracationic, and the uncharged (**H₂Tri**, **H₂TE**, and **H₂TF₅PhP**) compounds displayed negligible uptake. For *S. aureus*, the binding of the three compounds **cis-H₂DiE**, **trans-H₂DiE** and **H₂ME** corresponded to their PDI activity, but for the tricationic **H₂Tri** cell binding and bactericidal photoactivity did not match.

Based on results of their investigations with *para*-substituted porphyrin-based PSs, Alves and coworkers [11] concluded that there was no direct correlation between lipophilicity and bactericidal activity. Our results with *ortho*-substituted compounds confirm this observation. As shown in **Fig. 6A**, when the trans-dicationic isomer is excluded, the relationship between the photoefficacy against *E. coli* and the lipophilicity ($\log P_{ow}$) suggests that the highest PDT activity occurs within an intermediate range of lipophilicity. Compounds that were too hydrophilic (3^+ and 4^+ charges) or too lipophilic (0 and 1^+ charge) were less photoefficient. Excluding the dicationic **cis-H₂DiE** shifts the curve to the right, making the tricationic, less lipophilic **H₂TriE** the most efficient among the remaining PSs (**Fig. 5B**). This comparison demonstrates the strong effect of the position of positive charges on the PDI activity of the PSs. Shifting the *meso* pyridyl nitrogens from *para* to *ortho* position, made the compound with two adjacent positive charges the most photoefficient PS. Previous studies found that for the *para*-substituted porphyrins, the most photo-efficient against Gram-negative and Gram-positive microorganisms were the tricationic compounds [11, 67, 68], corresponding to the curve presented in **Fig. 6B**. With the *ortho*-substituted ethyl derivatives, however, we found that the photo-efficacy of the *trans* isomer (**Fig. 6C**) is strongly influenced by the differences in the outer wall and cytoplasmic membranes in Gram-positive and Gram-negative bacteria [51] and the concentration of the PSs. The apparent high PDI activity of the *trans* isomer at 0.1 μ M results not only from photo-inactivation but also from substantial dark toxicity (**Fig. 5C**). Similarly, the **cis-H₂DiE** also demonstrated high light-independent toxicity toward *S. aureus* at the same concentration (**Fig. 5B**). Given their amphiphilic character, this dark toxicity may reflect direct disruption of the bacterial plasma membrane at sufficiently high local concentrations.

Based on results obtained in this study it can be concluded that combining moderate lipophilicity (amphiphilicity) with the advantages of the *ortho* pyridyl nitrogen position [8, 16] and the two adjacent positive charges, is the way to design cationic PSs with high antimicrobial photodynamic activity.

Declarations

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Conflicts of Interest:

The authors declare no conflicts of interest.

Author Contribution

FS and WK conducted experiments. AT and LB conceived and designed research, analyzed data and wrote the manuscript.

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Data Availability

The experimental data that support the findings of this study are available in the Open Science Framework (OSF) at Storage as “Raw data” with the identifier <https://osf.io/phqad/files/osfstorage>

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Figures

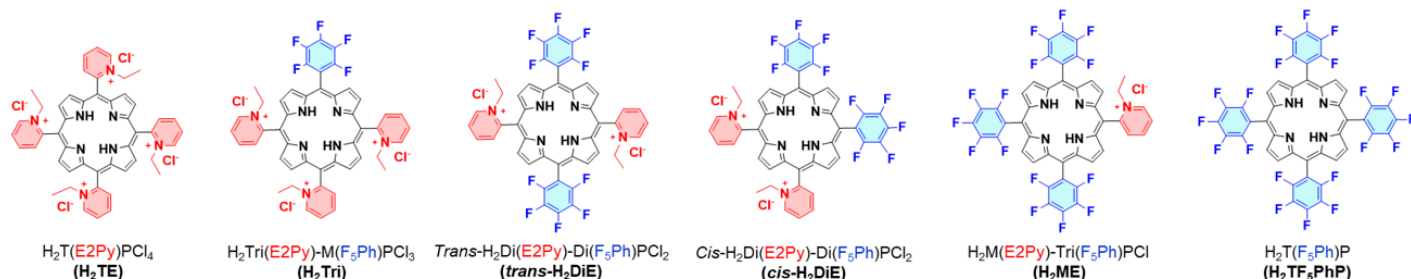


Figure 1

Structure of the tested cationic isomers of (*N*-ethylpyridinium-2-yl)-(pentafluorophenyl) porphyrins:

$\text{H}_2\text{T}(\text{E2Py})\text{PCl}_4$, $\text{H}_2\text{Tri}(\text{E2Py})\text{-M}(\text{F}_5\text{Ph})\text{PCl}_3$, $\text{cis-H}_2\text{Di}(\text{E2Py})\text{-Di}(\text{F}_5\text{Ph})\text{PCl}_2$, $\text{trans-H}_2\text{Di}(\text{E2Py})\text{-Di}(\text{F}_5\text{Ph})\text{PCl}_2$, $\text{H}_2\text{M}(\text{E2Py})\text{-Tri}(\text{F}_5\text{Ph})\text{PCl}$, $\text{H}_2\text{T}(\text{F}_5\text{Ph})\text{P}$.

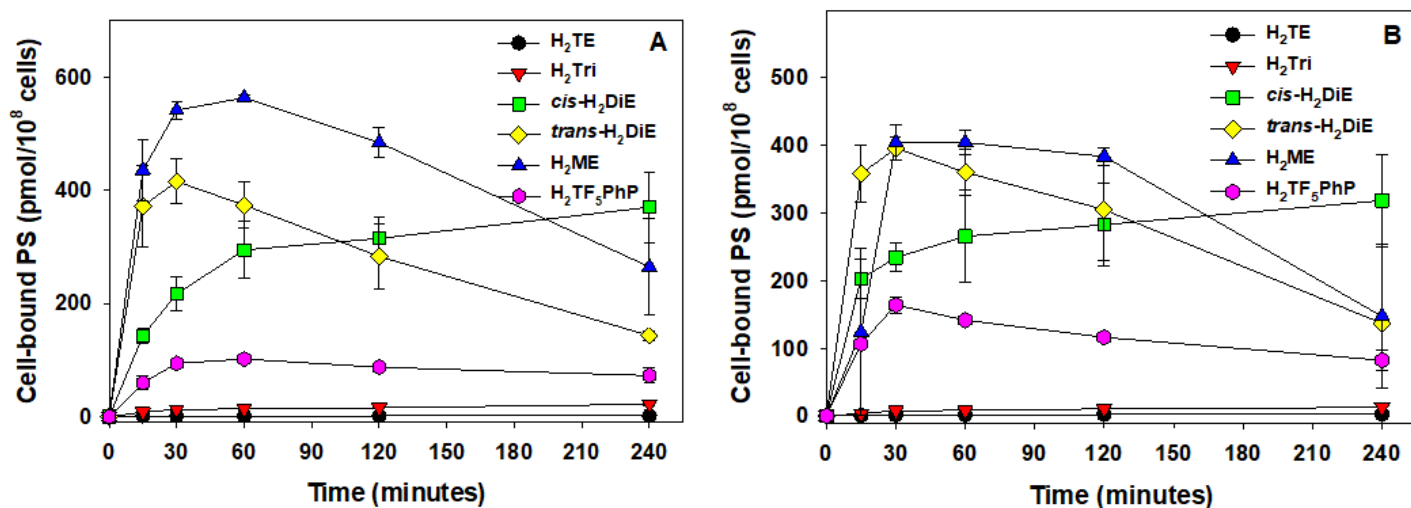


Figure 2

Time dependence of PSs cellular uptake. Stationary phase *E. coli* cultures were washed and resuspended in PBS glucose to $\sim 5 \times 10^8$ cells/ml. Aliquots were incubated in the dark at 37°C and 150 rpm with 5 μM of the tested compounds. At the indicated times 1.0 ml aliquots were transferred to Eppendorf tubes, rapidly washed with cold PBS and the pellet was resuspended in 2% SDS, 0.1 M NaOH

solution. Samples were incubated at room temperature in the dark for at least 24 hours or until transparent solutions were obtained. The amount of cell-bound PSs was determined by recording the emission fluorescence spectra (λ_{ex} at Soret band) **(A)** or the absorption spectra and calculating the area under the peaks **(B)**. PSs concentrations were estimated using separate standard curves for each compound. Cell number in each sample was determined by plating and counting colonies. Results of three independent experiments $\pm$ SD are presented.

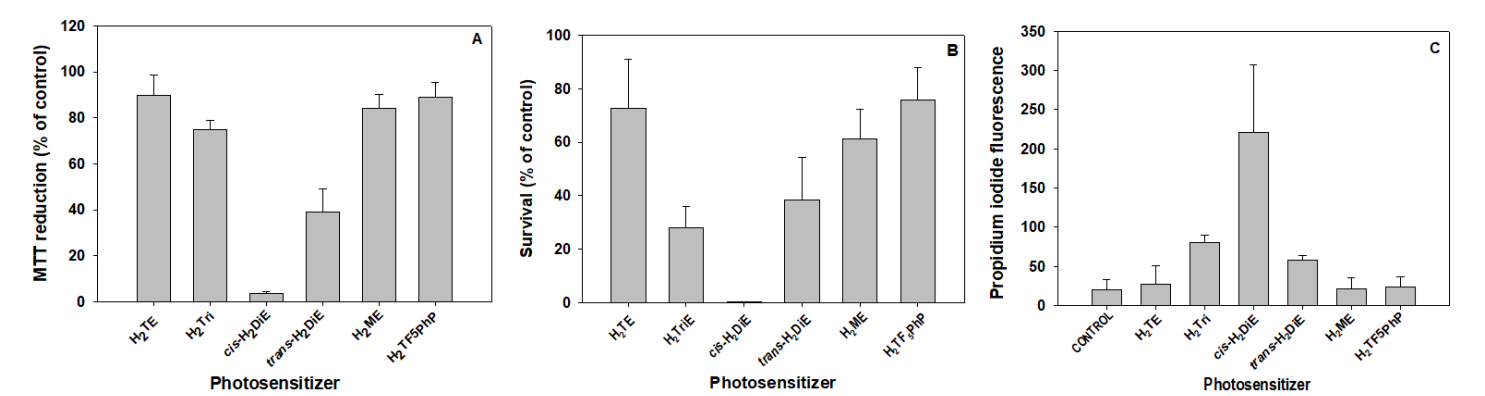


Figure 3

Photodynamic inactivation of *E. coli* treated with porphyrin-based photosensitizers. **(A)** Metabolic activity of *E. coli* following photodynamic treatment, assessed via the reduction of MTT to formazan. **(B)** Photodynamic inactivation efficacy, quantified by the reduction in colony-forming units (CFU) on agar plates. **(C)** Membrane integrity, evaluated by the uptake of propidium iodide (PI), a membrane-impermeable fluorescent dye. For all panels, *E. coli* cell suspensions (OD600 ~0.5) were incubated with 5 μ M of the tested PSs in PBS-glucose for 60 minutes in the dark. To eliminate the contribution of non-cell-bound photosensitizer, cells were thoroughly washed and resuspended in fresh PBS-glucose before 15 minutes of illumination at 64 mW/cm² (57.6 J/cm²). Immediately after the illumination, the respective assays were performed (details in “Materials and Methods” section). Results are expressed as the mean $\pm$ S.D. For panels (A) and (B), data were derived from three independent experiments performed in triplicate; for panel (C), data were derived from four independent experiments.

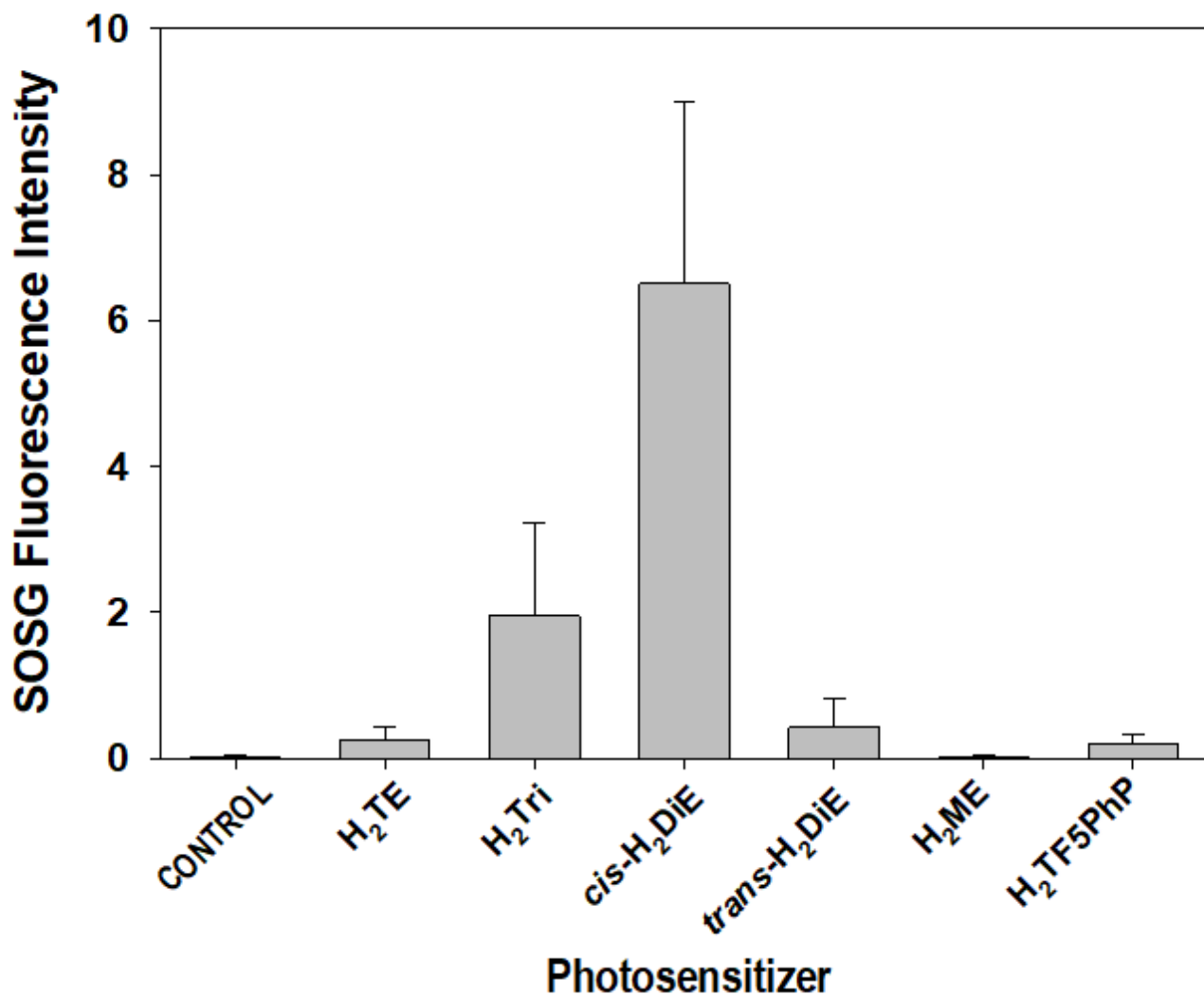


Figure 4

Fluorescence intensity of singlet oxygen sensor green (SOSG) in cells illuminated in the presence of the tested PSs. *E. coli* in PBS-glucose was incubated for 1 hour in dark with 5 μ M of the tested PSs. At the end of the incubation period SOSG was added to a final concentration of 10 μ M and samples were illuminated at 30 mW/cm² for 30 minutes. Confocal fluorescent images were obtained and SOSG fluorescent light emission by the cells was measured. Mean $\pm$ S.D. of three independent experiment is presented.

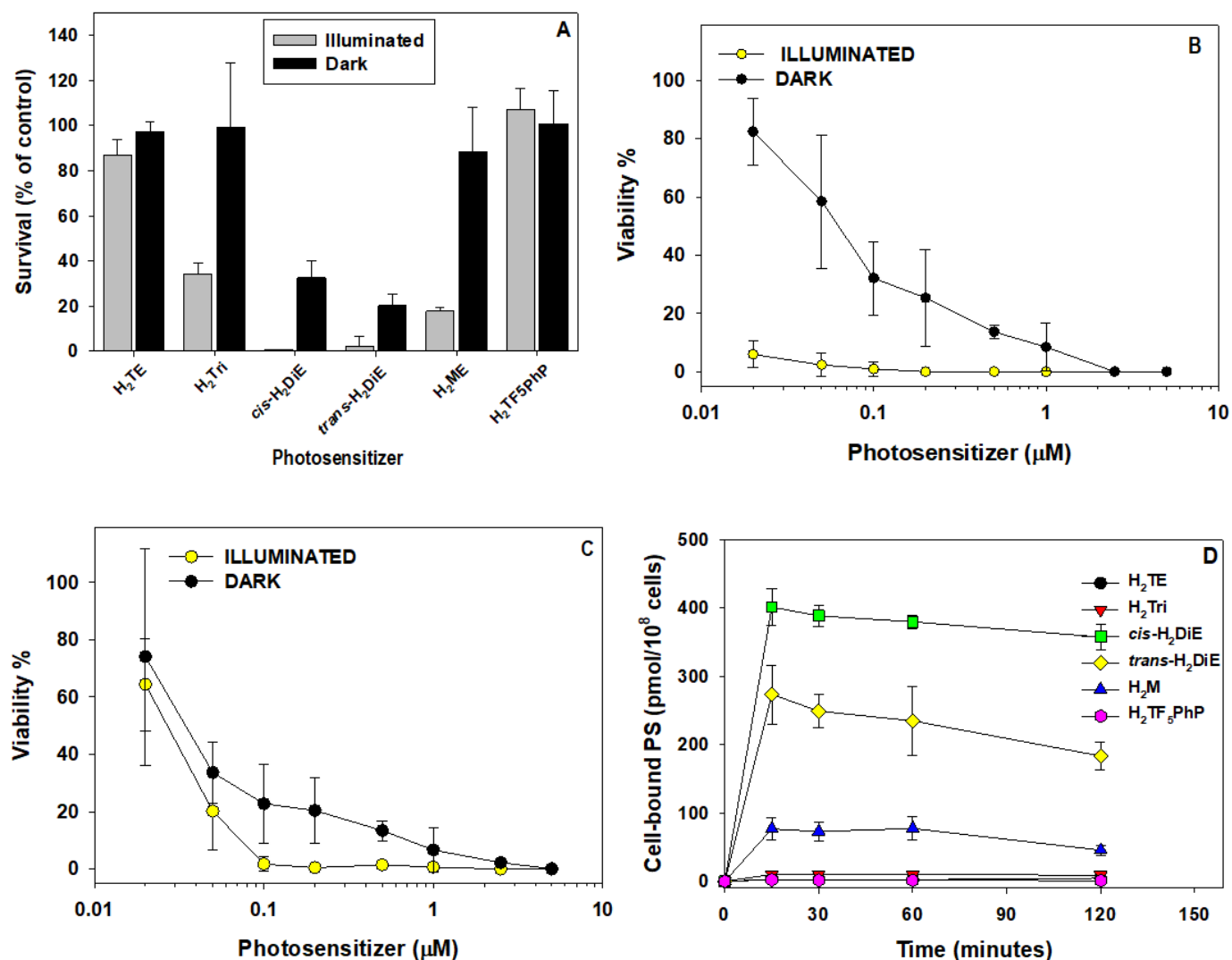


Figure 5

Phototoxicity, concentration-dependent efficacy, and cellular uptake kinetics of porphyrin-based photosensitizers in *S. aureus*. (A) Comparative light-dependent and dark toxicity of the tested PSs at 0.1 μM concentration. *S. aureus* suspensions were incubated with PSs for 1 hour, washed to remove unbound molecules, and subjected to 15 min of illumination at 64 mW/cm^2 (57.6 J/cm^2) or maintained in the dark using aluminum foil-wrapped controls. Survival was quantified via colony-forming unit (CFU) assays. (B, C) Concentration-dependent photodynamic and dark toxicity profiles for the *cis*-H₂DiE (B) and *trans*-H₂DiE (C) isomers, evaluated across a range of 0.02–5 μM , following the protocol described in (A). (D) Time-course analysis of PS uptake. *S. aureus* cells ($\sim 5 \times 10^8$ cells/ml) were incubated with 5 μM PS at 37°C and 150 rpm. At defined time points, aliquots were harvested, rapidly washed with ice-cold PBS, and lysed in a 2% SDS/0.1 M NaOH solution. After complete cell lysis, the concentration of internalized PS was determined by analyzing the absorption spectra and calculating the area under the peaks, calibrated against compound-specific standard curves. Cell densities were concurrently determined by

CFU to normalize the uptake data. All results are presented as mean $\pm$ S.D. of three independent experiments, with each sample processed in triplicate.

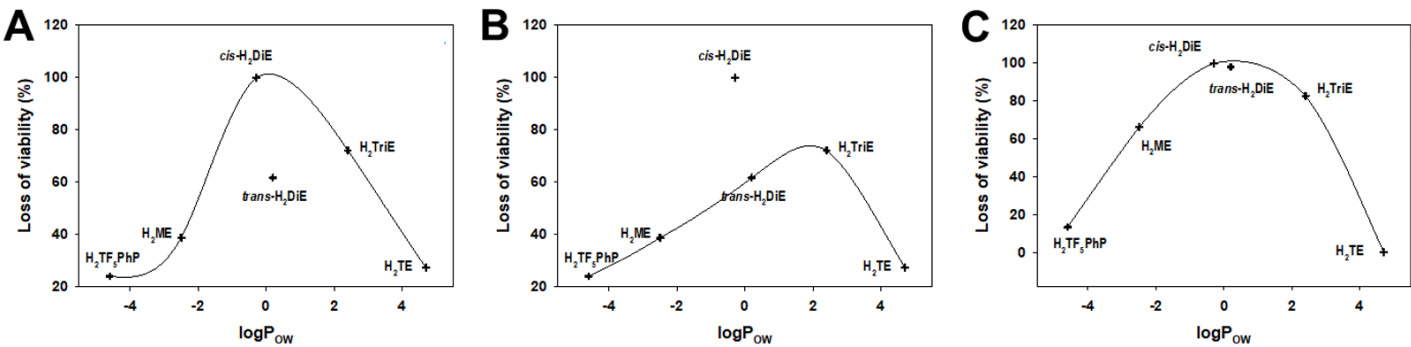


Figure 6

Relationship between lipophilicity ($\log P_{OW}$) and photodynamic activity of the tested PSs. A and B, *E. coli* (PSs at 5 μ M). Panel A, the dicationic *trans*-H₂DiE isomer excluded. Panel B, the dicationic *cis*-H₂DiE isomer excluded. Panel C, *S. aureus* (PSs at 0.1 μ M).