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Striking an access to the bacteria via (reversible) control of lipophilicity

Anzhela Galstyan,*[a] Johannes Putze,[b] Ulrich Dobrindt[b]

Abstract: Development of antimicrobial photodynamic therapy (aPDT) is highly dependent on the development of suitable photosensitizers (PS): ideally affinity of PS towards bacterial cells should be much higher than towards mammalian cells. Cationic charge of PS may lead to selective binding of PS to bacteria mediated by electrostatic interaction; however, the photodynamic outcome is highly dependent on the lipophilicity of PS. Herein we report the aPDT effect of silicon(IV)phthalocyanine derivatives bearing four positive charges and methyl, phenyl or naphthyl substituents on the periphery of the macrocycle. We show that via modulation of lipophilicity it is possible to find a therapeutic window where bacteria but not mammalian cells are effectively killed. The photobiological activity of these PSs dropped significantly when host-guest complexes of PSs with cucurbit[7]uril (CB[7]) were used. CB[7] blocks the hydrophobic part of the molecule and reduces lipophilicity of the PS, indicating that a hydrophobic interaction with the outer membrane of bacterial cells is essential for aPDT activity. Efficiencies of obtained PSs were evaluated using different uropathogenic *E. coli* isolates and human kidney epithelial carcinoma cells.

Introduction

Destruction of multidrug-resistant pathogens via reactive oxygen species (ROS) produced by photosensitizers (PS) of appropriate structure could represent a valuable alternative therapeutic modality addressing the problem of the increasing occurrence of infectious diseases.[1] Urinary tract infections (UTI) are the most common of all community-acquired bacterial infections in medical practice in industrialized countries, being the most common reason for antibiotic prescription.[2] This unfortunate excessive use of antibiotics has led to a considerable and alarming increase in the rate of resistant isolates in many countries.[3] Considering that the era of antibiotics will eventually come to an end, aPDT has been established as an effective antimicrobial strategy that doesn't rely on conventional antibiotic mechanisms.[4] Despite a number of advantages, there are limited studies where PDT was considered as a treatment option of bladder infections,[5] whereas within recent years, PDT of bladder cancer has found its way into widespread clinical use.[6] For clinical application of aPDT many characteristics of the used PS are important. Ideally, the PS should be able to accumulate in a high concentration in bacterial cells in a short time and be phototoxic only to bacteria with minimal or no damage to the healthy tissue. To fulfill these requirements conjugates with different bacteria targeting units were studied. Promising results have been obtained using antimicrobial peptide-PS conjugates.[7] Other studies have demonstrated that aPDT of individual pathogens is very effective when the PS is conjugated to a specific antibody.[8] Despite the vast amount of efforts directed toward the development of targeted aPDT agents, translation to clinical practice is rather slow due to a variety of unique challenges. These include: (i) high production costs that limit the wide use of these conjugates, (ii) the specificity of obtained conjugates to a particular bacterial strain, which might be advantageous in some cases, but non-beneficial when broad spectrum activity is desired.

Whereas Gram-positive bacteria could be effectively killed with neutral, cationic and anionic PSs, for inactivation of Gram-negative species only cationic PSs or non-cationic PSs with additional agents that permeabilize the outer membrane should be used.[9] Cationic PSs were shown to replace divalent cations of the bacterial surface that are responsible for the stabilization of lipopolysaccharide (LPS) and the native organization of the outer membrane. This replacement destabilizes the glycan coat leading to the "self-promoted" uptake of PSs.[10] Introduction of cationic charges promotes an association with the anionic surface of bacteria in contrast to the almost neutral membrane surface of healthy mammalian cells, giving a temporal selectivity for bacteria over mammalian cells. Indeed, many naturally occurring antibacterial peptides also have pronounced polycationic charge.[11] This approach was adopted by several groups to carry out aPDT with a large range of Gram-negative bacteria.[12] However, the cationic character is not the sole determinant required for the efficient photoinactivation process.[13] For instance, tetra- or octa cationic silicon(IV)phthalocyanines bearing four[14] or eight[15] *N*-methylated pyridoxy substituents in their periphery were proven to be inefficient for the photodynamic inactivation of Gram-negative *E. coli*. In our recent study, we show that boronic acid functionality can confer activity to the tetra cationic silicon(IV)phthalocyanine by substantially increasing the local concentration of PS.[16] Greater photobactericidal efficacy of toluidine blue in comparison to methylene blue was also found to be due to the stronger interaction of toluidine blue with LPS components.[17] Although aPDT can target both, external and internal, structures of bacteria, and does not really require the PSs to be internalized, factors that increase the amphiphilic character of the PS, such as the introduction of lipophilic groups or asymmetric charge distribution, can enhance the affinity of PSs for bacteria and increase their photocytotoxic effect.[18] These characteristics are associated with the ability of PSs to permeabilize the cell membrane, where the oxygen concentration is higher than in the aqueous medium.[19] Thus, the design and synthesis of PSs with cooperative behavior is a

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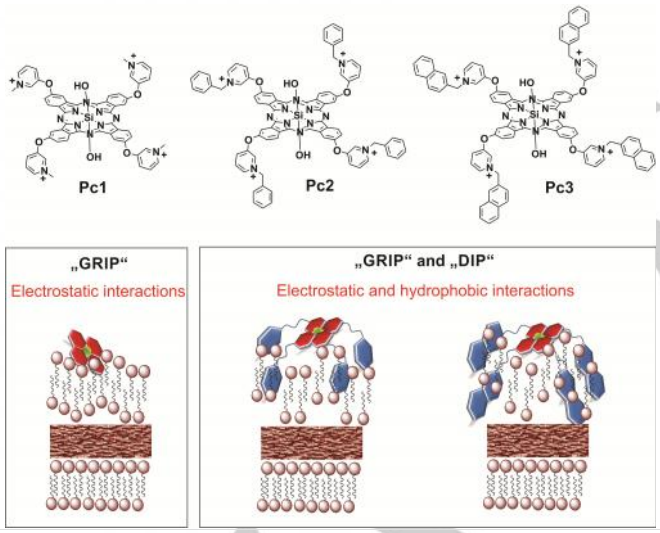
very successful approach: whereas positive charges enhance the ability to “grip” onto the cell membrane of Gram-negative bacteria, the lipophilicity of the PSs helps to penetrate the cell membrane and “dip” into sensitive parts of the bacterial cell. Tetrapyrrole-type photoensitizers carrying a moderate length of hydrophobic alkyl chains (C6 – C10) and a positive charge are found to be very effective towards Gram-negative bacteria.[20] Larger number of hydrophobic side groups/moieties also found to affect activity of phenothiazinium derivatives.[21] However, usually dark toxicity was observed when long alkyl chains were implemented in the structures leading to polarization and rupture of bacterial cell membranes in dark.[22] Recently, based on hydrophobic interaction an outer membrane-anchored aPDT nano agent was synthesized via conjugation of protoporphyrin IX and cholesterol.[23] The nano agent enables effective binding to the outer membrane of Gram-negative *E. coli*, resulting in a PS-enriched bacterial surface. Benefiting from the membrane accumulating ability of aromatic constituents Bazan *et al.* prepared a membrane-intercalating conjugated oligo electrolyte with high antibacterial capability upon irradiation.[24] Present evidence points out that PSs that exhibit high photobiological outcome are presented by dyes that besides positive charge have a well-balanced lipophilicity. Therefore, we introduced aromatic rings to the periphery of the non-active 2(3),9(10),16(17),23(24)-tetrakis-(3-pyridyloxy) phthalocyaninato dihydroxy-silicon (IV) to modulate its lipophilicity, to confer a photobiological activity and maximize the PDT outcome (Scheme 1).



Scheme 1. Schematic representation of probable disposition of the cationic silicon(IV)phthalocyanine molecules with different lipophilicity in the outer membrane of Gram-negative bacteria.

There is an emerging recognition of the role that supramolecular interactions play in tuning the properties of molecular species.[25] To elucidate the function of aromatic rings we used a supramolecular self-assembly-based approach that utilizes host-guest interaction between PS and CB[7]. CB[7] is a water soluble donut-shape molecule possessing a hydrophobic cavity. It is able to make host guest complexes with hydrophobic molecules in aqueous media [26] and was widely used as a drug carrier. Several research groups have developed antibacterial agents that are able to assemble and disassemble with CB[7] and its close analogs. However, whereas in some cases bactericidal properties appeared to be activated upon formation of supramolecular assemblies,[27] the activity of other agents was significantly reduced upon host-guest complexation.[28] Generally, the intrinsic efficacy of PSs was enhanced and a synergistic effect was expected, however, the biological output of this supramolecular interaction of a macrocyclic host and the PS was not always additive.

Although several factors that are important in the photodynamic efficiency of silicon(IV)phthalocyanines (SiPc) have already been described before,[16,29] they have not been consistently evaluated. In an effort to clarify this, a comprehensive study was undertaken to determine the impact of the lipophilicity and host-guest complexation on the molecular features of 2(3),9(10),16(17),23(24)-tetrakis-(3-pyridyloxy) phthalocyaninato dihydroxy-silicon (IV) and its aPDT efficacy. The results of this study provide a better understanding of the structure–property relationships of the PSs and may provide guidelines to develop more efficient and selective antimicrobial agents.

Results and Discussion

Synthesis and photophysical characterization: The reactions used to prepare a series of SiPcs are given in the Experimental Section and in the Scheme S1 of the Supporting Information. The phthalocyanine macrocycle was synthesized via condensation of diiminoisoindoline according to previously published methods.[16,30] Quaternization of the pyridoxy substituents on the non-peripheral positions of the macrocyclic core using dimethylsulfide, benzyl bromide, and 2-(bromomethyl) naphthalene led to the formation of SiPcs containing methyl (**Pc1**), phenyl (**Pc2**) and naphthyl (**Pc3**) substituents, correspondingly. The proposed structures of the newly synthesized Si(IV)Pcs were confirmed by 1D and 2D NMR spectroscopy, high-resolution electrospray ionization mass spectrometry (HRMS) and fourier transform infrared (FT-IR) spectroscopy.

Phthalocyanine derivatives used in this study bear hexacoordinate silicon (IV) in the central cavity, which is coordinated to the four ring nitrogen atoms of the phthalocyanine central cavity and two hydroxyl groups. Situated at axial positions hydroxyl substituents can disrupt direct π-π stacking of the macrocycles, compiling favorable photophysical characteristics to the PS. Their electronic absorption and basic photophysical data were measured in *N,N*-dimethylformamide (DMF) as well as in water (H_2O) and data are summarized in Table 1.

Absorbance maxima in the UV-Vis spectra of all PSs correspond well with previously published data.[16,30] In DMF an intense sharp Q-band at around 672 nm is followed by a weaker at 604 nm. In water solution, their absorption spectra are also dominated by sharp Q band with 5-7 nm bathochromic shifts of the maxima.

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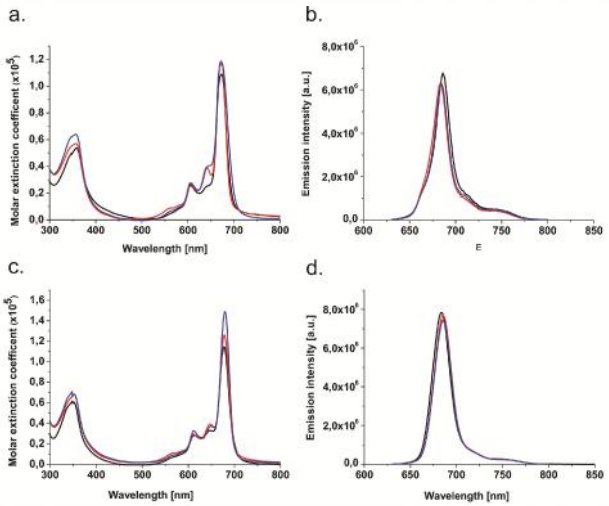

Figure 1. UV/Vis absorption and fluorescence spectra of **Pc1** (black), **Pc2** (red) and **Pc3** (blue) in water (a and b) and *N*,*N*-dimethylformamide (c and d).

Whereas in DMF molar absorption coefficients were rather similar, in water media an increase of ε_{max} for **Pc2** and **Pc3** was observed (Figure S1, Supporting Information). These differences may reflect the different arrangement of the fluorophores dictated by the bulkiness of the substituents. Fluorescence emission was recorded upon excitation at 610 nm. All PSs emit strongly in the near-infrared in DMF and H_2O with λ_{max} of around 685 nm (Figure 1 and Table 1).

Table 1. Electronic absorption and photophysical data for **Pc1, Pc2** and **Pc3.**

PS solvent	λ_{abs}/nm (log10ε)	λ_{em}/nm	c_F[b] ± 0.03	c_Δ[c] ± 0.03	log$P_{o/w}$ PS/PS(CB[7])$_4$
Pc1 DMF	673 (5.05) 604 (4.43) 358 (4.74)[a]	686	0.43	0.52	-
Pc1 H_2O	678 (5.04) 611 (4.43) 351 (4.77)[a]	684	0.37	0.17	-0.98[a]/-1.03
Pc2 DMF	672 (5.06) 604 (4.44) 357 (4.76)	684	0.41	0.59	
Pc2 H_2O	678 (5.10) 610 (4.46) 352 (4.83)	685	0.36	0.22	-0.71/-1.08
Pc3 DMF	672 (5.07) 604 (4.45) 357 (4.82)	684	0.39	0.56	
Pc3 H_2O	679 (5.16) 611 (4.51) 349 (4.82)	685	0.31	0.25	-0.24/-0.73

[a]Data from ref 16. [b]Quantum yields were calculated by the steady state comparative method using zinc phthalocyanine as a reference (c_F = 0.28 in DMF). [b]Quantum yields were measured using the relative method using zinc phthalocyanine (c_F = 0.56) or methylene blue (c_Δ = 0.52) as a reference.

The fluorescence quantum yields (Φ_F) of the SiPcs were calculated by the steady state comparative method using zinc phthalocyanine (ZnPc) as a reference [31] and reported in Table 1. Φ_F values of all SiPcs in water were found to be slightly lower than in DMF: their values ranged from 0.31 to 0.37 in H_2O vs 0.39 to 0.43 in DMF. This shows that the aqueous medium is not inducing significant aggregation of the PSs and hence does not quench their excited state properties (Table 1).

Singlet oxygen is commonly considered the main species taking part in PDT, and thus PSs with higher singlet oxygen quantum yields (Φ_Δ) are usually regarded as the more promising ones. The obtained PSs were assessed for their singlet oxygen generation in DMF and H_2O. Φ_Δs were measured by the relative method,[32] using 1,3-diphenylisobenzofuran (DPBF) in DMF and 9,10-anthracenyl-*bis*(methylene)dimalonic acid (ABMDMA) in H_2O, as a reactive trap for photogenerated $^1O_2$ and excitation with visible light filtered below 610 nm. The concentration of the scavenger (DPBF) was monitored spectroscopically at 414 nm in function of the irradiation time of the PS, from which the values of Φ_Δ were determined. Non-substituted ZnPc was used as a reference compound (Φ_Δ^{ZnPc} = 0.56 in DMF).[33] A slight increase of Φ_Δ value was observed for **Pc2** and **Pc3** in comparison to **Pc1** (Figure S3, Supporting Information). A quantitative analysis of photooxidation reactions leading to the loss of emission of the water-soluble anthracene 9,10-dipropionic acid was used to measure $^1O_2$ production in aqueous media. Methylene blue was used as a reference compound (Φ_Δ^{MB} = 0.52 in H_2O).[34] The **Pc2** and **Pc3** were found to be better singlet oxygen generators, but the efficiency is still comparable for all PSs (Figure S4, Supporting Information). Relevant photophysical data are given in Table 1.

Addition of four equivalents of CB[7] to the aqueous solution of **Pc1** did not cause any noticeable change in the absorption spectrum of the latter. For **Pc2** and **Pc3** addition of CB[7] resulted only in a slight change of the absorption maxima and hypochromicity, indicating that the PSs are in a monomeric state and the binding of CB[7] did not contribute to the aggregation-disaggregation process and consequently in the change of photophysical characteristics (Figure S2, Supporting Information). On the contrary to our results, PSs that easily form aggregates based on hydrophobic interactions were shown to disaggregate upon addition of CB[7].[27b] In these cases, the formation of supramolecular PSs improves photophysical characteristics, especially singlet oxygen quantum yields that contribute to increase in antibacterial efficacy of PSs. Further, we explored the impact of substituent and formation of host-guest complexes with CB[7] on the lipophilicity of the PS. Highly hydrophilic PS will dissolve well in aqueous media and remain in the extracellular space. Replacing the methyl group with phenyl or naphthyl substituents may enable the PS to partition into the lipid bilayer of the cell's membrane. However, it is important to find a right balance, since too hydrophobic molecules tend to aggregate in the aqueous phase and subsequently quench production of singlet molecular oxygen. The octanol-water partition coefficient (log P) is often quoted to give an indication of the lipophilicity of a drug. The values are determined for all PSs and for PS(CB[7])$_4$ supramolecular complexes and are given in

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Table 1. The obtained values indicate that the partitioning into the lipid phase increases, as expected, in the following order **Pc1** < **Pc2** < **Pc3**. Supramolecular encapsulation of the PSs by CB[7] results in the shifts of the logP values to negative values for **Pc2** and **Pc3**. This clearly shows that CB[7] blocks hydrophobic aromatic constituents resulting into more hydrophilic PS.

***In vitro* photodynamic activities:** A UTI usually starts as a bladder infection (cystitis) but can develop into an acute kidney infection (pyelonephritis). In some cases, UTI can result in urosepsis, a disease with a mortality rate of 20-40% in the setting of optimal resuscitation in wealthy countries. More than 85% of all UTIs are caused by *Escherichia coli* [35] – Gram-negative bacteria which are commonly less susceptible to aPDT treatment.[9]

Uropathogenic *E. coli* strains UTI89 (cystitis isolate), 536 and CFT073 (pyelonephritis isolates) were used to determine the most efficient PS of the series. Bacterial suspensions with bacterial numbers of approx. $1x10^9$ CFU mL^{-1} were incubated with 1 µM solutions of the corresponding PS for 15 min before unbound PS was removed. The binding of the PSs to the bacterial cells was determined by measuring the concentration of the PS that remained in the culture supernatant after removing the bacteria by centrifugation (Figure 2). All PSs and their supramolecular complexes with CB[7] show comparable binding to the bacterial cells. This is probably due to the same amount and position of the charged groups. However, uptake/binding of PSs does not appear to correlate with their antimicrobial efficacy (vide infra).

The antimicrobial activity of **Pc1, Pc2** and **Pc3** was evaluated by the determination of the number of viable colony forming units (CFU) per milliliter. Under irradiation with polychromatic light of a projector lamp (>610 nm, 10 mW/cm^2) the viability of microorganisms was not affected by light alone - light control, nor by the direct effect of the PS - dark control (Figure S4, Supporting Information).

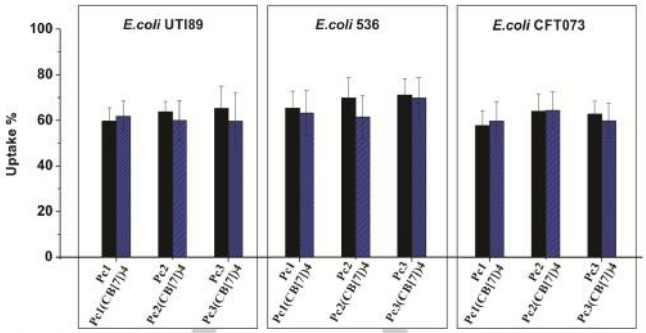

Figure 2. The binding/uptake amounts of photosensitizers used in this study by bacterial cells after 15 min incubation with 1µM solution. Mean ± SD is presented.

To investigate the influence of the light fluence on the PDT activity different exposure times that correspond to light doses of 9, 18 and 36 Jcm^{-2} were used. The photoinactivation of *E. coli* using a 1µM solution of PS is shown in Figure 3. Although the cationic charge of **Pc1** promotes electrostatic interaction with the negatively charged outer surface of the bacterial cells, it did not cause any significant reduction in bacterial numbers. In contrast, **Pc2** and **Pc3** showed significant reduction of CFU mL^{-1} upon light activation, but to a different extend. Whereas **Pc3** reduced the survival of all *E. coli* strains by >log5 units at 1 µM concentration and 36 Jcm^{-2} light exposure, the reduction of bacterial survival caused with **Pc2** was lower under the same conditions. Once accumulated on the surface of the bacteria, an important aspect of triggering the antibacterial properties is the accessibility of the PS to the hydrophobic and oxygen-rich membrane interior where the concentration of dissolved oxygen is 2–4 times higher than in the extracellular space.

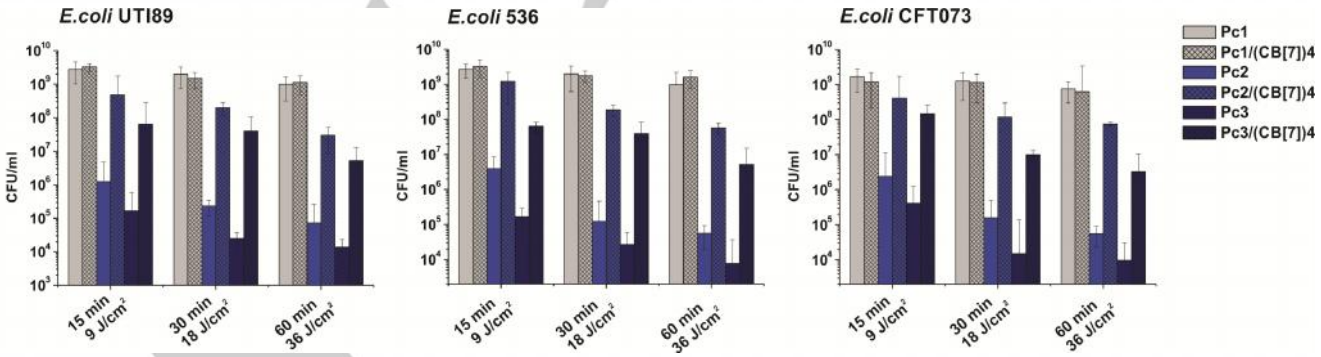

Figure 3. Bacterial photoinactivation with 1 µM solution of Pc1, Pc2, Pc3 and their supramolecular complexes with CB[7]. Dose effect in light-induced toxicity against E. coli 536, E. coli CFT073 and E. coli UTI89. Mean ± SD is presented.

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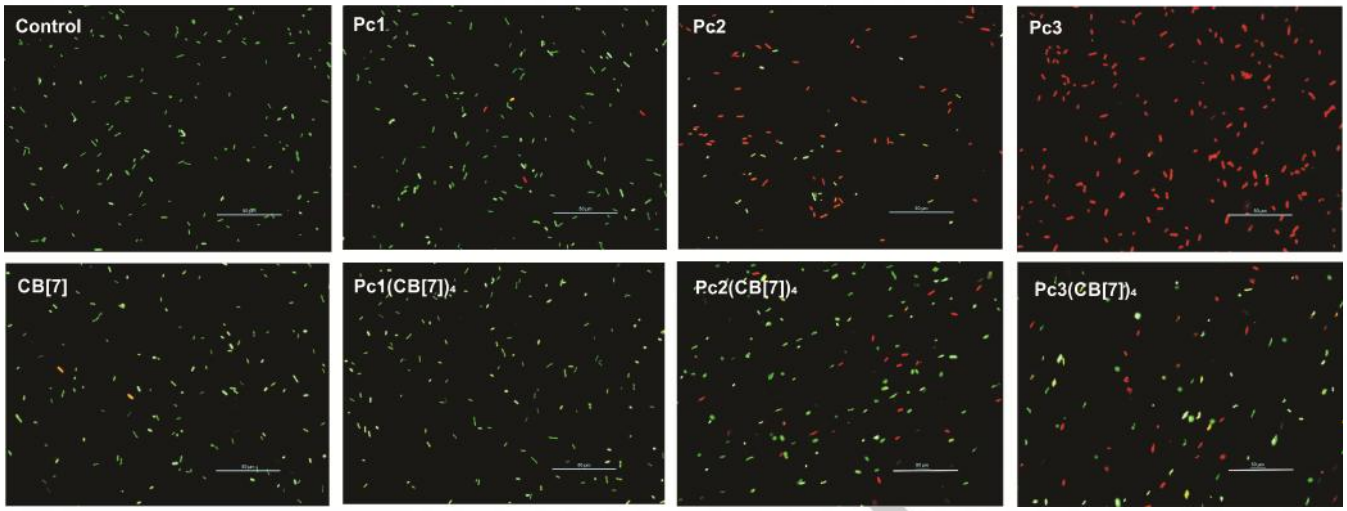

Figure 4. Representative fluorescence images of LIVE/DEAD assayed *E. coli* UTI89 after treatment with PS, CB[7] only or PS(CB[7])$_4$ complex and 3 min of irradiation. Scale bar: 50 μM.

After light excitation membrane components of the bacterial cells become damaged when the PS is inserted into the membrane. When the PS is less deeply bound to the membrane interface it will be less active, since the concentration of the oxygen in the extracellular interior is low and the larger fraction of the generated singlet oxygen will be deactivated before interacting with and oxidizing membrane components. Most likely **Pc1** is too hydrophilic and its interaction with the outer membrane of Gram-negative bacteria is only of electrostatic nature. The photokilling effect of **Pc2** and **Pc3** was strongly reduced when CB[7] was present in the solution. Presumably, a supramolecular structure consisting of PS(CB[7])$_4$ combination generated via the intermolecular non-covalent association, "blocks" hydrophobic interactions with the outer cell membrane of bacteria. This confirms that the observed antibacterial effects of **Pc2** and **Pc3** are a direct consequence of primary damage to the highly sensitive cellular targets in the bacterial membrane. Irrespective of the fact that the PS(CB[7])$_4$ accumulated to high levels on the bacterial outer membrane, it did not exhibit higher phototoxic efficiency, since the generation of ROS occurs on the site that is less likely to lead to the cell death. Similar imbalance between the binding of a PS and its photo-efficiency had already been reported for the benzylidene cyclopentanone based system.[36] It was shown that despite comparable Φ_Δ values and very similar binding/uptake of cationic and anionic derivatives to bacterial cells, the latter showed better aPDT activity. The authors suppose that the synergy effects of concise chemical structure and suitable logP value endow the good aPDT effect. In their report Hamblin *et al.* tested poly-L-lysine chlorin(e6) conjugates for inactivation of *E. coli* and show that despite higher uptake of the 8-lysine conjugate in comparison to the 37-lysine conjugate, only the latter was able to kill effectively Gram-negative bacteria at low concentrations.[37] The authors conclude that a certain length of the polycationic chain is necessary to allow the PS to enter the outer membrane of *E. coli.* In contrast, the large polycationic carrier is not necessary or even disadvantageous for the inactivation of Gram-positive bacteria such as *S. aureus*.[37] Our results are also in accordance with the previously published studies showing that the photosensitizing activity of cationic porphyrins towards Gram-negative bacteria is reduced by their incorporation into liposomes, most likely due to the hindered interaction of PS with cell binding sites.[38] **Pc2** and **Pc3** can partly retain their photosensitizing activity after formation of host-guest complexes with CB[7] indicating that PS still could be displaced from the supramolecular complex and be extracted into the bacterial cell membrane. The photokilling efficacy of studied PSs was further confirmed by the Live/Dead assay using *E. coli* strain UTI89. As seen from Figure 4 when bacteria were incubated with **Pc1**, CB[7] or **Pc1(CB[7])$_4$** complex mostly viable, Syto 9 (green) stained cells were observed. When **Pc2** and **Pc3** were used a high proportion of propidium iodide stained (red) dead cells can be seen. As expected, the viability of bacteria was increased when **Pc2(CB[7])$_4$** and **Pc3(CB[7])$_4$** were utilized, further confirming our results obtained by CFU counting. The fact that the observed photoinactivation rates correlate with the lipophilicity of PSs rather than photophysical characteristics or cell binding extent indicates that the PS localization is a dominant factor. Though positive charge-induced electrostatic interaction contributes greatly to the binding of a cationic PS onto the negatively charged bacterial cell wall, enhancement of its hydrophilic character may restrain further entry of the PS into the bacterial cell membrane. This consideration should weigh heavily in optimizing the design of PSs.

In order to rationally select the most suitable PS, it is important to study the photocytotoxicity of all PSs when they are in contact with mammalian cells. It is easy to assume that the lipophilicity will also affect the uptake of PSs by mammalian cells and compromise their targeting specificity.[39] Viability assays using **Pc1**, **Pc2** and **Pc3** were carried out using the human kidney epithelial cell line A-498 as a model of the host cells that might be damaged during the PDT treatment of UTI.

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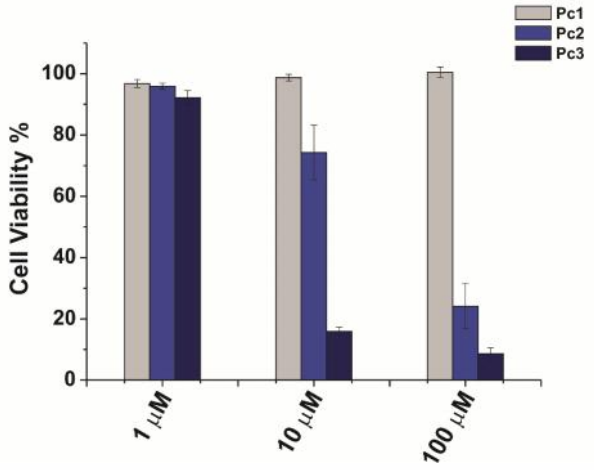

Figure 5. Photocytotoxic effect of **Pc1, Pc2,** and **Pc3** on A498 cells incubated with different PS concentrations (1 -100 μM) for 1h and irradiated for 1h (36 J/cm^2). Mean ± SD is presented.

By irradiation with 36 J/cm^2 of NIR-light, no damage of A-498 cells was induced when **Pc1** was used, that had been incubated at concentrations of 1-100 μM for 1h. More lipophilic **Pc2** and **Pc3** were found to be photocytotoxic only at concentrations at least one order of magnitude higher than those causing significant bacterial cell death. At low concentration (1μM) almost no photodamage of mammalian cells was observed (Figure 5). Thus, the optimal combination of PS concentration and drug-light interval can enable selective inactivation of bacterial cells.

Conclusions

In this work, we performed a comprehensive study showing that a positive charge of highly hydrophilic silicon(IV)phthalocyanine does not necessarily ensure its antimicrobial efficacy. Readily accessible, lipophilic analogs were prepared via quaternization of pyridine moieties on the macrocyclic ring. We show that Gram-negative uropathogenic *E.coli* strains are killed by irradiation with NIR light when the used PS can translocate through the bacterial membranes. Lipophilicity of the PSs was modulated via host-guest complexation with CB[7]. Using an optimal combination of PS concentration and drug-light interval a substantial phototoxic effect could be achieved against bacteria, whereas mammalian cells remained unaffected under the same conditions. We believe that this analysis may help to understand which factors should be considered when developing an improved PDT protocol and also reinforces the need of new, more effective PSs.

Experimental Section

General: Synthetic procedures were carried out under dry argon atmosphere unless otherwise specified. All reagents and solvents were purchased at the highest commercial quality available and used without further purification unless otherwise stated. Column chromatography was carried out on silica gel Merck-60 (230–400 mesh, 60 A°), and TLC on aluminum sheets pre-coated with silica gel 60 F254 (E. Merck). NMR spectra were recorded on an ARX 300 or an AMX 400 from Bruker Analytische Messtechnik (Karlsruhe, Germany) spectrometer at a constant temperature of 298 K. ^{1}H NMR chemical shifts δ are given relative to TMS (d = 0) and referenced to the solvent signal. Electrospray ionization (ESI) mass spectra were recorded on a Bruker Daltonics (Bremen, Germany) MicroTof with loop injection.

Synthesis: The starting 4-(3-pyridyloxy)phthalonitril[40] and 4-(3-pyridyloxy)-1,3-diiminoisoindoline[41] were synthesized and purified by known procedures. The further reactions towards 2(3),9(10),16(17),23(24)-tetrakis-(3-pyridyloxy) phthalocyaninato dihydroxy-silicon (IV) followed the modify pathway of synthesis described previously.[30] **Pc1** was synthesized according to the ref. 16. **Pc2**: 2(3),9(10),16(17),23(24)-tetrakis-(3-pyridyloxy) phthalocyaninato dihydroxy-silicon (IV) (50 mg, 0.053 mmol) and benzyl bromide (25 μl, 0.212 mmol) were dissolved in dry dimethylformamide and the mixture was stirred at 50°C overnight. After cooling to rt, CH_2Cl_2 was added to the solution to precipitate the product. It was dissolved in DFM and precipitated with CH_2Cl_2 again and the precipitate was dried under reduced pressure. Yield: 94%. 1H NMR (600 MHz, DMSO-d6/MeOD): δ, ppm: 9.75–9.63 (m, 8H), 9.48-9.39 (m, 4H), 9.29-9.19 (m, 4H), 8.67-8.63(m, 4H), 8.39-8.31 (m, 8H), 7.80-7.69 (m, 8H), 7.51-7.44 (m, 12H), 6.09 (s, 8H). 1H,1H GCOSY (600 MHz / 600 MHz, DMSO-d6/MeOD) [selected traces]: δ1H / δ1H: 9.73/6.02; 9.19/6.02; 8.64/6.02; 7.69/6.02; 7.48/6.02; 7.70/7.50; 7.80/7.49; 9.74/8.38; 9.65/8.34; 9.47/8.39; 9.40/8.36; 9.19/8.33; 8.65/8.33; 9.74/920. HRMS m/z: Calcd. for $C_{80}H_{58}N_{12}O_6Si$ $[M]^{4+}$: 327.60880, found: 327.60874; $C_{80}H_{58}N_{12}O_6SiBr$ $[M]^{3+}$: 463.78458, found: 463.78476. FT-IR: 745; 1083; 1272; 1497; 1585; 3032; 3400. **Pc3**: 2(3),9(10),16(17),23(24)-tetrakis-(3-pyridyloxy) phthalocyaninato dihydroxy-silicon (IV) (70 mg, 0.074 mmol) and 2-(bromomethyl) naphthalene (65 mg, 0.295 mmol) were dissolved in dry dimethylformamide and the mixture was stirred at 50°C overnight. After cooling to rt, CH_2Cl_2 was added to the solution to precipitate the product. It was dissolved in DFM and precipitated with CH_2Cl_2 again and the precipitate was dried under reduced pressure. Yield: 87%. ^{1}H NMR (600 MHz, DMSO-d6/MeOD): δ, ppm: 9.78 – 9.61 (m, 8H), 9.49-9.43 (m, 4H), 9.16-9.11 (m, 4H), 8.72-8.61 (m, 4H), 8.38-8.25 (m, 8H), 7.91-7.54 (m, 12H), 7.47-7.40 (m, 16H), 6.11-5.85 (m, 8H). 1H,1H GCOSY (600 MHz / 600 MHz, DMSO-d6/MeOD) [selected traces]: δ1H / δ1H: 9.62/5.99; 9.31/5.85; 9.13/5.99; 7.71/6.10; 7.64/ 5.99;9.70/8.33; 9.61/8.66; 9.60/9.11; 9.43/8.37; 9.35/8.36; 8.71/8.35; 8.62/8.27; 8.32/8.12; 7.91/7.64; 7.72/7.44; 7.65/7.45. HRMS m/z: Calcd. for $C_{96}H_{66}N_{12}O_6SiBr$ $[M]^{3+}$: 530.47251, found: 530.47248. FT-IR:755; 1077; 1268; 1468; 1577; 3013; 3361.

Partition coefficient: 1-Octanol/water partition coefficients ($\log P_{o/w}$) were determined at 25 °C using equal volumes of water (1 mL) and 1-octanol (1 mL). The final concentration of compound was approx. 20 μM. The mixture was stirred for 1 h and centrifuged (10 min, 4400 rpm) to enable a phase separation. An aliquot (50 μL) of aqueous and organic phases were dissolved in 1 mL of *N,N*-dimethylformamide (DMF) and final concentration determined by absorption spectroscopy. $\log P_{o/w}$ was calculated according to the following equation:

$$\log P_{o/w} = \log ([PS]_{octanol} / [PS]_{water})$$

Photophysical measurements: Absorption spectra were measured on a Varian Cary 500 or Agilent 8453 spectrophotometer and baseline corrected. Steady-state emission spectra were recorded on a HORIBA Jobin-Yvon IBH FL-322 Fluorolog 3 spectrometer equipped with a 450 W xenon-arc lamp, double-grating excitation and emission monochromators (2.1 nm/mm dispersion; 1200 grooves/mm). All solvents used were of the spectrometric grade.

Fluorescence quantum yields: Fluorescence quantum yields (Φ_F) were determined by a comparative method using the following equation:

$$\Phi = \Phi_s \cdot (n/n_s) \cdot (F/F_s) \cdot (A_s/A)$$

Where *F*= are areas under fluorescence emission curves of the Pc1, Pc2, Pc3 and F_s of ZnPc as standard. *A* and A_s are the absorbances of the samples and standard at the excitation wavelength and *n* and n_s are refractive indices of solvents used for samples and standard, respectively. ZnPc in DMF was used as a standard, Φ_F=0.28.[31] for the determination of fluorescence quantum yields. The sample and the standard were both excited at the same relevant wavelength (610 nm). The absorbance of the solutions at the excitation wavelength ranged between 0.05 and 0.1.

Singlet oxygen quantum yields: Singlet oxygen quantum yields were determined using the relative method. Polychromatic irradiation from a projector lamp passing through a cut-off filter at 610 nm was used to carry out the experiments. Freshly prepared dye solution in dark flasks were mixed with the PSs only immediately before taking the samples at "0 time." $^1O_2$ photogeneration rates in DMF were derived using 1,3-diphenylisobenzofuran (DPBF). The initial absorbance of DPBF was adjusted to about 1.0, then the PS was added to reach absorbance about 0.2-0.3. The photooxidation of DPBF was monitored between 0 s to 25 s. $^1O_2$ photogeneration rates in water were derived using 9,10-anthracenediyl-*bis*(methylene)dimalonic acid (ABMDMA) as a fluorescent monitor (λ_{exc} = 370 nm) for photosensitized bleaching rates. The c_Δ for the samples was calculated according to the following equation:

$$\Phi_\Delta^S = \Phi_\Delta^R \cdot \frac{r_S}{r_R} \cdot \frac{\int_{\lambda_1}^{\lambda_2} I_0(\lambda) \cdot (1-10^{-A_R(\lambda)}) \cdot d\lambda}{\int_{\lambda_1}^{\lambda_2} I_0(\lambda) \cdot (1-10^{-A_S(\lambda)}) \cdot d\lambda}$$

where *r* is the slope of the monitor's bleaching over time (plotted as ln (F/F_0)), $\lambda_1 - \lambda_2$ is the irradiation wavelength interval, $I_0(\lambda)$ the incident spectral photon flow, $A(\lambda)$the absorbance, and the subscripts *R* and *S* are the reference (zinc phthalocyanine c_Δ = 0.56 in DMF[33] and methylene blue, c_Δ = 0.52 in H_2O[34]) and sample, respectively. The incident intensity can be approximated by a constant value, drawn out of the integral and canceled. All recorded graphics are shown in Supporting Information (Figure S2 and S3).

Bacteria culture conditions and *in vitro* photodynamic inactivation: The *E. coli* strains were maintained on Luria-Bertani (LB) broth agar and were stored at 4 °C. A single isolated colony was picked from this plate, transferred in 3 ml LB broth and incubated aerobically at 37 °C overnight in a rotary incubator with shaking at 180 rpm (rotations per minute). On the next day, the bacteria were suspended in 10 ml of fresh LB medium to an optical density (OD_{600}) of 0.1 and grown in a flask to an OD_{600}=0.4. The bacterial suspension was then centrifuged at 4000 rpm for 5 min, resuspended in PBS to the final bacterial concentration of ca. $1x10^9$ cells per mL and subsequently used for experiments. To induce $^1O_2$ generation the 1 ml PS stained bacteria (15 min, 37 °C) were placed in 24-well plate and were irradiated with polychromatic light of a projector lamp passing through a 610 nm cut-off filter (10 mW/cm^2) for 15 min, 30 min and 60 min from the top of the plate. After irradiation, the living bacterial cells were determined by serial dilutions of the bacterial suspension and plating on LB agar plates. The plates were incubated overnight at 37 °C and the number of CFU/mL was determined. As controls, PS treated bacteria were kept in the dark (dark control) and untreated bacteria were exposed to light (light control).

Binding affinity of PSs to the bacterial cells: The binding affinity (BA) of PS was measured using indirect spectrophotometrical measurements of the *E. coli* suspensions. The samples and their blanks were incubated in the dark for 15 min and were centrifuged for 5 min at 4000 rpm. An aliquot of the supernatant (1 ml) was then transferred to a cuvette and the absorbance was measured. BA, i.e. the proportion of Pc adsorbed to the bacterial cells was expressed as a percentage from the total Pcs.

$$BA = 100\text{-}(ABS_{sample}/ABS_{blank} \times 100)$$

Live/Dead staining: Cells were treated in the same way as for the evaluation with CFU counting. Following irradiation, samples were incubated with LIVE/DEAD® bacterial viability staining kit (*BacLight™ Bacterial Viability Kit,* Invitrogen L13152) for 15 min according to manufacturer's instructions. A 10 µL aliquot of the mixture was added to glass slides and covered with a glass coverslip. Fluorescence images were taken with a Nikon Eclipse Ci microscope, (40 X magnification). Excitation at 480 nm and long pass filter were used.

Cell line, growth condition and *in vitro* viability assay: The human kidney epithelial cell line A-498 was cultured in Eagle´s Minimal Essential Medium (EMEM) supplemented with 10 % fetal bovine serum (FBS), 2 mM L-glutamine, 1 mM sodium pyruvate and 1 x non-essential amino acids at 37°C in a humidified 5% CO_2 incubator. About 2×10^4 A-498 cells per well in the medium were incubated in 96-well plates and allowed to adhere overnight. Photosensitizers were examined in the concentration range of 0 – 100 µM. Cells were incubated for 1 h at 37 °C in the dark; subsequently, the medium was discarded and replaced with new medium followed by an illumination of 1 h. The Alamar Blue assay was used to investigate the cell viability: After illumination, the cells were incubated at 37°C under 5 % CO_2 for 24 h. The medium was replaced with 200 µL of 10 % Alamar Blue (Sigma) solution in growth medium followed by incubation for 3 h. The plate was shaken on a microplate reader (Tecan, Switzerland) for 20 s before the fluorescence at each well was measured (Ex 535 nm / Em 595 nm). The viability of A-498 cells was then expressed as the relative viability (% control).

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Keywords: phthalocyanine • photodynamic therapy • antimicrobial agents • *E. coli* • host-guest interaction

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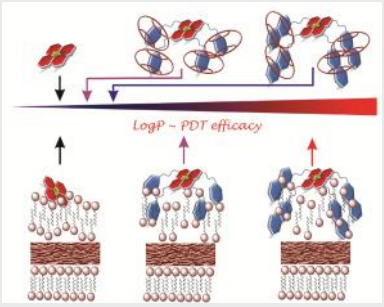
FULL PAPER

Location matters: The approach described in this paper yields photosensitizers that exhibit high photocytotoxic effect against gram-negative *E.coli* at low concentration without harming eukaryotic cells. The effectivity correlates with the lipophilicity of the photosensitizer that changes upon complexation with CB[7].



Dr. A. Galstyan, Dr. J. Putze, Prof. Dr. U. Dobrindt

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Striking an access to the bacteria via (reversible) control of lipophilicity