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Approaches to selectivity in the Zn(II)–phthalocyanine-photosensitized inactivation of wild-type and antibiotic-resistant *Staphylococcus aureus*

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A number of Zn(II)–phthalocyanines bearing peripheral substituents of cationic nature due to the presence of quaternarized anilinium or ammonium groups were shown to be efficient photoantimicrobial agents: a 4–5 log decrease in the survival of both wild-type or methicillin-resistant *Staphylococcus aureus* was obtained upon short irradiation times in the presence of phthalocyanine concentrations as low as 0.1 μM. A careful selection of the experimental protocol, and in particular the use of short (5 min) incubation times and mild irradiation parameters, allowed one to achieve a high selectivity of *S. aureus* photoinactivation as compared with important constituents of potential host tissues, such as human fibroblasts and keratinocytes. The efficiency and selectivity of the photoprocess were not affected by the presence of 5% human serum.

Introduction

The increasing diffusion of infectious diseases represents a major challenge for human health worldwide, especially as a consequence of the continuous emergence of antibiotic-resistant bacteria [1,2] even in nosocomial environments,[3] the large number of antibiotic-induced adverse side effects,[4] and the possible transmission of infectious diseases to humans from animals and other sources.[5] Therefore, the development of alternative therapeutic modalities based on different strategies is being actively pursued. In this connection, photodynamic therapy (PDT) can represent a useful approach, particularly for the treatment of localized infections.[6] Typically, porphyrins [7,8] and phthalocyanines [9,10] have been shown to photosensitize, when irradiated with visible light, a variety of microbial pathogens, including Gram-positive and Gram-negative bacteria, fungi, yeasts and mycoplasmas; most importantly, the antimicrobial effect of photodynamic agents appears to be independent of the antibiotic sensitivity of the treated pathogens.[11] So far, photodynamic modalities have been proposed for the sterilization of contaminated blood,[12] as well as for the treatment of infected wounds [13] and periodontal [14] infections.

A widespread clinical application of PDT obviously requires that the photocytotoxic action against microbial cells takes place with minimal damage to the host tissues. In actual fact, photoactivated porphyrin derivatives promote the efficient inactivation of mammalian cells both *in vitro* and *in vivo*.[15] Therefore, it appears necessary to define phototherapeutic protocols which lead to an extensive and highly preferential decrease in the survival of microbial cells.

For some years, our laboratories have been carrying out a systematic investigation on the relationships between the chemical structure and the antimicrobial photoactivity of phthalocyanines (Pcs); such research has enabled us to identify a restricted number of Pcs which exhibit a very high photosensitizing efficiency against both wild and antibiotic-resistant strains of bacteria and yeasts.[14] In this paper, we show that a suitable selection of experimental parameters to be used for the PDT treatment guarantees a satisfactory level of selectivity for microbial cells in comparison with fibroblasts and keratinocytes, which are among the main constituents of host tissues, such as skin and mucosas.

Materials and methods

Chemicals

All the phthalocyanines (Pcs) used in the present investigation were prepared by chemical synthesis in the laboratories of Molteni Farmaceutici (Forence, Italy), solubilized in dimethyl sulfoxide (DMSO) at a concentration of 1 mg ml^{-1}, aliquoted and stored at −20 °C. The synthetic procedures and the methods used for the characterization of the Pcs are described elsewhere.[16] The concentration of the Pcs was routinely determined from the molar extinction coefficient which was determined in dimethylformamide (DMF) at 680 nm (corresponding to the λ_{max}).

Sodium dodecyl sulfate (SDS) was obtained from Merck. All other chemicals and solvents were commercially available reagents of analytical grade.

Bacterial strains

Staphylococcus aureus strain 25923 ATCC, and methicillin-resistant *Staphylococcus aureus* MRSA 110 were grown aerobically at 37 °C in brain heart infusion (BHI, Difco, Detroit, Michigan). The bacterial cells in the stationary phase of growth were harvested by centrifugation of broth cultures (2000*g* for 15 min), washed twice with 10 mM phosphate-buffered saline (PBS) and diluted in the same buffer to an optical density of 0.7 at 650 nm, corresponding to 10^8–10^9 cells per ml.

Cell lines and culture conditions

HT-1080, a tumour cell line derived from human fibrosarcoma,[17] and HaCaT, which are spontaneously transformed

and non-tumorigenic human keratinocytes,[18] were used as model cells. The cell lines were cultured in Dulbecco's modified minimal essential medium (D-MEM, Sigma St. Louis, Missouri) containing 10% heat-inactivated fetal calf serum (FCS, Boehringer, Mannheim, Germany) and supplemented with 100 units per ml of penicillin, 100 μg ml^{-1} of streptomycin, 0.25 μg ml^{-1} of amphotericin (Sigma) and 2 mM of glutamine (Sigma). The cell lines were routinely checked for the absence of mycoplasma contamination.

Phthalocyanine binding studies with microbial cells

Samples containing 10^8–10^9 cells per ml prepared by dilution from a previously washed bacterial suspension in PBS at pH 7.2 were incubated in the dark for 5 min with phthalocyanine concentrations ranging from 0.1–2.5 μM in PBS + 5% DMSO. The cells were then recovered by centrifugation and the pellets were dissolved in 2% aqueous SDS (1 ml). After 18 h incubation at room temperature, each SDS-treated sample was divided into two portions: 0.4 ml were stored for the determination of the protein content by the bicinchoninic acid (BCA) assay,[19] and 0.6 ml were used to evaluate the phthalocyanine concentration after a suitable dilution in 2% SDS. The fluorescence emitted in the 650–800 nm spectral range upon excitation at 610–630 nm was measured for each sample with a Perkin Elmer MPF4 spectrophotofluorimeter, and the Pcs concentrations were calculated by interpolation with a calibration plot previously obtained with known concentrations of each phthalocyanine in 2% SDS solution.

Phthalocyanine binding studies with human cells

Approximately 5×10^5 HT-1080 and HaCaT cells were seeded in 25 cm^2 tissue culture flasks in D-MEM medium. After 24 h the medium was changed to PBS + 5% DMSO containing the photosensitizing drug. The accumulation of Pcs by the cells was studied as a function of the phthalocyanine at concentrations ranging from 0.1 to 2.5 μM. The cells were incubated for 5 min at 37 °C in a dark humid atmosphere containing 5% CO_2. At the end of the incubation period, the medium was removed and the cell monolayer was carefully washed five times with cold PBS containing Ca^{2+} and Mg^{2+} ions. Then, 1 ml of 2% aqueous SDS was added to each flask. After magnetic stirring for 1 h, each sample was divided into two portions: 0.2 ml were stored for the determination of the protein content by the bicinchoninic acid assay, and 0.8 ml were analyzed fluorimetrically in order to measure the amount of the phthalocyanine. The uptake of Pcs by cells was expressed as nmol of photosensitizer per mg of protein.

Irradiation experiments and survival studies with microbial cells

Suspensions (50 μl) of *S. aureus* ATCC 25923 and MRSA 110 containing approximately 2×10^8 CFU per ml in PBS were transferred into wells of a microtitre plate. The same volume of a phthalocyanine solution in PBS containing 10% DMSO was added to each well to a final phthalocyanine concentration ranging from 0.01–10 μM. Control studies showed that no effect on bacterial cell survival was induced by the PBS + 5% DMSO medium. Samples were incubated at 37 °C in the dark for 1–5 min., then irradiated with a Waldmann lamp (Schwenningen, Germany) whose emission was filtered to isolate the 600–700 nm light. The irradiations were performed for 1–5 min at a fluence rate of 10–50 mW cm^{-2}. Other photosensitization studies were performed in the presence of 5% amounts of human serum. At the end of the irradiation experiments, unirradiated and irradiated bacterial cells were serially 10-fold diluted with the growth medium and the number of colonies found after 18–24 h incubation at 37 °C were counted.

Human cell photosensitization studies

For the photosensitization studies 1×10^5 cells were seeded in 35 mm diameter culture dishes containing 2 ml of D-MEM with 10% FCS. After 24 h the culture medium was removed, the subconfluent cells were incubated for 1–5 min in the dark at 37 °C with 0.1–2.5 μM phthalocyanine solutions in PBS + 5% DMSO and irradiated with 600–700 nm light (10–50 mW cm^{-2}) for 1–5 min. Again, the presence of 5% DMSO did not affect the cell survival. At the end the cells were trypsinized, and the cell survival was determined by the trypan blue exclusion test. Parallel photosensitization studies were performed in the presence of 5% amounts of human serum.

Results and discussion

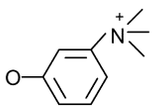
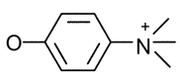
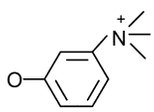
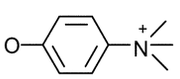
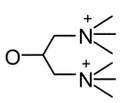
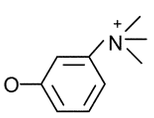
Preliminary cell photosensitization studies

The photobactericidal activity of Zn(II)–phthalocyanines has been found[16,20] to be markedly influenced by their chemical structure. However, very little is known about the selectivity of the phthalocyanine derivatives for their prospective use in clinical applications. In order to identify those structural features which yield an optimal combination of efficient antibacterial action with a high selectivity for microbial cells in comparison with human cells, we examined a selection of phthalocyanine derivatives differing in the number (one, four or eight) of substituents protruding from the peripheral positions of the tetraazaisoindole macrocycle, as well as in the location of the substituents in the α or β position of the benzene ring (see Table 1). Only Pcs which had a cationic character owing to the presence of quaternary nitrogen atoms were used, since positively charged photosensitizers have been shown to exhibit a particularly high phototoxicity against bacteria.[14,16,21] As can be seen from the results presented in Table 1, all the Pcs tested by us induced a 4 to 5 log decrease in the survival of both the wild and the methycillin-resistant *S. aureus* strains after 5 min exposure to light at a fluence rate of 50 mW cm^{-2} in the presence of photosensitizer concentrations as low as 0.1 μM, *i.e.* by using relatively mild experimental conditions. However, in the case of an octasubstituted phthalocyanine (RLP073), the dose had to be increased to 2.5 μM in order to achieve similar decreases in survival. Interestingly, the overall photoefficiency was very similar for Pcs bearing different numbers of the aromatic functional group, as well as for trimethylammonium-phenoxy derivatives where the quaternary nitrogen is in the meta or para position of the benzene ring. Therefore, it appears that factors, such as the orientation of the peripheral substituents, do not play an important role in the photosensitization of bacteria by Zn(II)–phthalocyanines.

Our data support the hypothesis[21,22] that the driving force behind such photoprocesses is the presence of positively charged moieties which establish an electrostatic interaction with negative groups at the surface of the outer wall: as a consequence of which, selected components of the wall are modified in the initial stages of the photoprocess, resulting in an increase in the wall permeability and an accelerated influx of photosensitizer molecules to inner cellular domains.[11,22] The final binding of phthalocyanines with cells is however regulated by additional factors such as the degree of hydrophobicity which favours a tight association with the cytoplasmic membrane.[6] This explains the greater accumulation of monocationic as compared with tetracationic phthalocyanines.

With few exceptions, the photosensitivity level of MRSA was essentially identical to that typical of the wild strain. This observation is in agreement with previous results (see ref. 7 and 11) indicating that the efficiency of the photosensitizing action of Pcs on bacteria is independent of their antibiotic resistance spectrum. On the other hand, the photosensitivity of HT-1080 and HaCaT was appreciably affected by the number of peri-

Table 1 Decrease in survival (in log) of *S. aureus* cells and fibroblasts upon 5 min irradiation with 600–700 nm light (50 mW cm^{-2}) after 5 min dark incubation with 0.1 μM Zn(II)–phthalocyanine bearing different numbers of substituents in the α or β position of the isoindole rings.

Phthalocyanine	R	*S. aureus* ATCC 25923	*S. aureus* MRSA 110	Fibroblasts
MRLP126 α mono		4.3	3.2	2.6
MRLP166 α mono		4.4	4.6	5.5
RLP068 α tetra		4.6	4.6	0.3
MRLP164 α tetra		4.6	4.6	0.1
[a]RLP035 β tetra		5.2	5.1	5.5
[a]RLP073 octa		5.5	5.5	5.5

[a] The dose used was 2.5 μM.

Table 2 Recovery of phthalocyanines (nmol mg^{-1} of cell protein) from *S. aureus* cells and fibroblasts after 5 min incubation with 0.1–2.5 μM photosensitizer concentration

Phthalocyanine	Dose/μM	*S. aureus* ATCC 25923	*S. aureus* MRSA 110	Fibroblasts HT-1080
RLP068	0.1	1.17 ± 0.09	1.09 ± 0.06	0.19 ± 0.02
	0.25	2.57 ± 0.09	2.58 ± 0.03	0.33 ± 0.01
	0.5	5.10 ± 0.12	4.32 ± 0.12	0.54 ± 0.03
	1.0	8.70 ± 0.73	7.46 ± 0.18	0.90 ± 0.06
	2.5	12.44 ± 0.36	11.50 ± 0.54	1.28 ± 0.11
MRLP164	0.1	1.60 ± 0.03	1.51 ± 0.05	0.63 ± 0.02
	0.25	3.39 ± 0.02	2.98 ± 0.55	1.28 ± 0.10
	0.5	5.82 ± 0.21	5.57 ± 0.25	2.11 ± 0.06
	1.0	11.06 ± 0.11	9.98 ± 0.17	3.73 ± 0.15
	2.5	16.32 ± 0.41	13.54 ± 1.46	5.77 ± 0.30

pheral substituents (see Table 1). Thus, octacationic Pcs, such as RLP035 and RLP073, showed a photoinactivating efficiency which was comparable with that observed for these Pcs against *S. aureus*. A minimal drop of fibroblast survival took place when the irradiation was performed in the presence of tetracationic Pcs, namely RLP068 and MRLP164. Therefore, these two Pcs were selected for further more detailed investigations.

In all cases, control cells, which were treated with 0.1 μM Pcs and were not exposed to light, underwent no detectable decrease in the number of colony-forming units.

Differential affinity and photosensitivity of bacterial and human cells toward selected phthalocyanines

As mentioned in the Introduction, PDT is most likely used for the treatment of localized microbial infections. Therefore, in our parallel studies on the sensitivity of bacterial and human cells to red light irradiation in the presence of RLP068 and MRLP164, we envisaged a possible scenario where the *in situ* number of *S. aureus* cells per unit volume (10^8 cells ml^{-1}) was 2 to 3 orders of magnitude larger than that of fibroblasts or keratinocytes (10^5–10^6 cells per ml). Under these conditions, the amount of phthalocyanine accumulated by *S. aureus* after 5 min incubation steadily increased with increasing photosensitizer concentration over the 0.1–2.5 μM range (see Table 2). In all cases, no appreciable increase in the amount of *S. aureus*-bound phthalocyanine was observed upon prolonging the incubation up to 60 min. Thus, it is likely that a plateau value for the amount of cell-associated photosensitizer was reached after 5 min incubation. Such an equilibrium value might have been obtained even after incubation times as short as 1 min, as suggested by the essentially identical degree of photosensitivity exhibited by the two *S. aureus* strains when irradiated after 1 or 5 min incubation (Table 3). A precise measurement of the amount of cell-bound RLP068 cannot be performed for practical reasons. Apparently, only minor differences were noticed for the localization and affinity of the two Pcs between the wild and the methycillin-resistant *S. aureus* strains. No attainment of a plateau value for the concentration of cell-bound phthalocyanine was observed. On the other hand, MRLP164 displayed a slightly higher affinity for the two *S. aureus* strains especially at the 1.0 and 2.5 μM concentrations.

Clearly, also HT-1080 human fibroblasts accumulated increasingly larger photosensitizer amounts as the phthalocyanine concentration in the incubation medium was progressively increased (Table 2). Without reaching a plateau value, in this case, MRLP164, which has a para-*N*,*N*,*N*(trimethyl) ammoniumphenoxy side chain, exhibited a significantly larger

Table 3 Decrease in survival (in log) of bacterial and human cells upon irradiation for 5 min with 600–700 nm light after dark incubation with 0.1 μM phthalocyanine

	RLP068				
	50 mW cm^{-2}				10 mW cm^{-2}
Cell lines	1 min inc.	5 min inc.	5 min inc.[a]	5 min inc. + 5% HS[b]	5 min inc.
ATCC 25923	4.5	4.6	3.4	4.6	3.8
MRSA 110	4.0	4.6	3.2	4.5	3.3
HT-1080	0	0.3	0	0	0
HaCaT	—	0	—	0	—

[a] 1 minute irradiation. [b] HS = human serum.

	MRLP164				
	50 mW cm^{-2}				10 mW cm^{-2}
Cell lines	1 min inc.	5 min inc.	5 min inc.[a]	5 min inc. + 5% HS[b]	5 min inc.
ATCC 25923	3.2	4.6	3.2	4.6	3.8
MRSA 110	2.6	4.6	2.7	4.6	3.1
HT-1080	0	0.1	0	0	0
HaCaT	—	0.2	—	0.1	—

affinity for fibroblasts as compared with the meta-substituted analogue RLP068. A comparative analysis of the phthalocyanine recovery data from bacterial and human cells showed that under our experimental conditions *S. aureus* accumulated 2- to 3-fold larger amounts of MRLP164 at all the phthalocyanine concentrations tested by us. On the contrary, in the case of RLP068, this ratio increased from about 6 to almost 10 for incubations with 0.1 μM and respectively, 2.5 μM phthalocyanine. Therefore, we carried out some photosensitization studies with the two *S. aureus* strains and fibroblasts at different RLP068 concentrations in order to assess whether the variations in the amount of cell-bound phthalocyanine could be correlated with differences in cell photosensitivity. The experiments were extended to human keratinocytes (HaCaT) to test the general validity of the information thus obtained.

The results of these experiments are shown in Fig. 1a. It appears that a very high selectivity of bacteria photoinactivation was achieved upon irradiation with 0.1 μM phthalocyanine: an almost 5 log decrease in survival of wild-type and methicillin-resistant *S. aureus* was observed after 5 min irradiation, while only a barely detectable decrease in the survival of fibroblasts and keratinocytes took place. A good selectivity of bacteria photoinactivation was also obtained in the presence of 0.25 and 0.5 μM RLP068, whereas both types of human cells were extensively inactivated with 1 or 2.5 μM phthalocyanine. Quite similar results were obtained when MRLP164 was used as the cell photosensitizer (Fig. 1b). Thus, the differential photosensitivity between *S. aureus* cells and fibroblasts or keratinocytes appears to be controlled by the amount of cell-bound phthalocyanine: a significant degree of phototoxicity to human cells is induced upon binding of about 4 and 1.3 nmol mg^{-1} of endocellular protein for MRLP164 and the more efficient RLP068, respectively. Such values are 4- to 6-fold larger than those required to cause a drastic drop in the survival of bacterial cells. It is likely that fibroblasts and keratinocytes, due to their size which is substantially larger compared to that of Gram-positive bacterial cells, require a sufficiently high density of phthalocyanine molecules in order to undergo irreversible photodamage leading to cell death.

Lastly, we investigated whether the selectivity of the bacteria photoinactivation process would be affected by changing specific experimental parameters. In particular, in view of *in vivo* applications of this photosensitization modality, we checked the effect of milder irradiation conditions, such as lower fluence-rates, as well as shorter irradiation or incubation

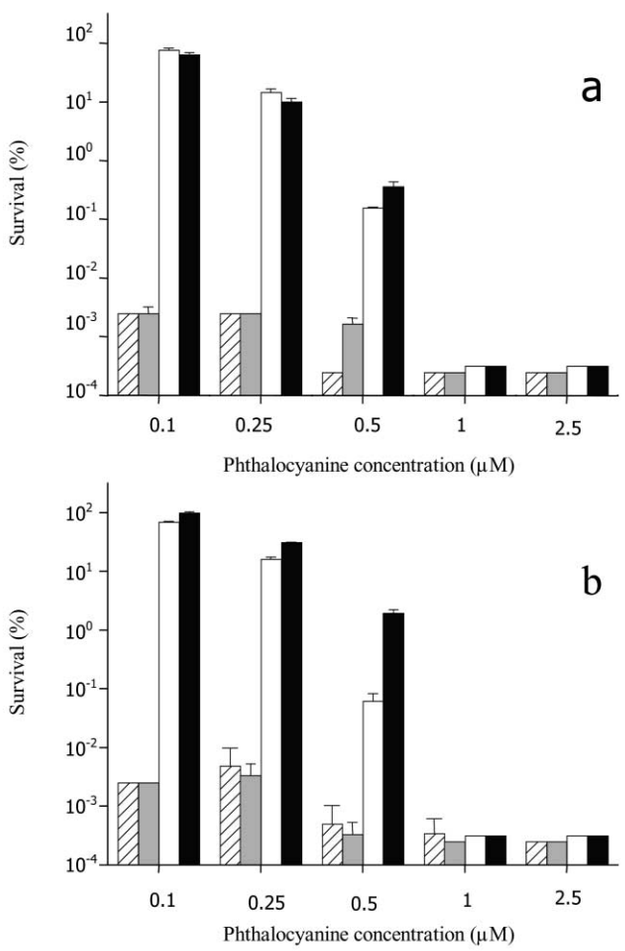


Fig. 1 Survival of *S. aureus* cells (▨; ATCC 25923, ▩; MRSA 110), fibroblasts (□) and keratinocytes (■) upon 5 min irradiation with 600–700 nm light (50 mW cm^{-2}) after 5 min dark incubation with 0.1–2.5 μM of RLP068 (a) and MRLP164 (b) in PBS + 5% DMSO.

times. The data thus obtained are summarized in Table 3. Clearly, the milder protocols did not influence the already low photosensitivity of fibroblasts and keratinocytes, while some decrease in the efficiency of bacteria photoinactivation occurred, especially in the case of MRLP164. It is important to underline that the photosensitising activity of the two

phthalocyanines was not appreciably affected by the addition of 5% human serum (Table 3). This finding is certainly positive since serum proteins are known to form stable complexes with several phthalocyanines,[23] thereby lowering their efficiency as photosensitizers of cells and tissues.[24]

Conclusions

Our results suggest that a careful control of the experimental parameters allows one to achieve a highly preferential phototoxicity of selected visible light-activated phthalocyanines against bacterial pathogens. In particular, by using RLP068 and MRLP164 an extensive inactivation of *S. aureus* cells occurs at much lower phthalocyanine concentrations and light doses than those required to damage important constituents of host tissues, such as fibroblasts and keratinocytes. In this connection, the present findings support previous observations[25] showing that the photosensitised killing of *Helicobacter pylori* with toluidine blue could be obtained by using less drastic irradiation conditions than those causing an important damage of rat gastric mucosa. One advantageous feature of the antibacterial PDT protocol is certainly represented by the short (less than 5 min) incubation time between the cells and the photosensitising agent which is necessary to have a 4–5 log decrease in the pathogen population. In general, mammalian cells develop a significant level of photosensitivity only after an at least 30–60 min incubation with porphyrin-type photosensitizers.[15] Of course, our present results are valid for cells in suspensions. If our approach involving a short irradiation time (5 min) and low fluence rate (50 mW cm^{-2}) is effective also for animal models, a phototherapeutic protocol with favourable features for clinical applications would be available. Moreover, the apparent lack of inhibitory effect of serum on the efficiency of the overall photoprocess involving RLP068 and MRLP164 opens the way to the use of such phthalocyanines for the phototreatment of infected wounds and burns where PDT could have a distinct improvement over several out of the presently adopted therapeutic modalities.[26]

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