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Zinc phthalocyanines-mediated photodynamic therapy induces cell death in adenocarcinoma cells

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Abstract

A panel of eight Zn-phthalocyanines (Zn-Pcs) (**1**)–(**8**), differently substituted on the benzo units, was synthesized either by direct cyclic-tetramerization of substituted phthalonitriles (compounds **1** and **2**), or leading from the easily available tetrasulphonyl phthalocyanine to yield the sulfonamido derivatives **3** and **4**, or else via the chloromethylation of precedent Zn-Pc followed by reaction with nucleophiles affording the dicationic Zn-Pcs (**5**) and (**6**) or the neutral Zn-Pcs (**7**) and (**8**). The phototoxicity of these new compounds was evaluated in vitro on human colon adenocarcinoma cell line (HCT116), and their effect compared with those induced by porfimer sodium. The results are reported as IC_{50} values, following exposure of the cells to different Zn-Pcs concentration and irradiation with a 500 W tungsten/halogen white lamp. The cationic Zn-Pc (**5**) and (**6**) together with the Zn-Pc (**7**), featuring 12 methoxy groups, were found good or fairly good photosensitizers while the more lipophilic Zn-Pcs (**1**)–(**4**) and (**8**) were found devoid of activity.

Keywords: Zn-phthalocyanines; Photosensitizers; PDT; Tumor cells

1. Introduction

Photodynamic therapy (PDT) is becoming widely accepted as a potential treatment for many forms of cancer, curing early or localized disease and improving the quality of life in advanced disease and increasing survival [1]. PDT involves three main components, a photosensitising compound (PS), visible light, and molecular oxygen that in turn will become the prime component of selective tumour cell destruction [2]. After administration of a PS, which should be preferentially retained by tumor cells, the subsequent irradiation with visible light of the cancer tissues only allows specific inactivation of neoplastic cells [3,4]. Treatment efficacy is strictly related to the presence of oxygen, and two basic types of reaction can occur following PS photoactivation. One mechanism involves free radical generation (type I photochemical reaction) while the other results in production of singlet oxygen, O_2 ($^1\Delta_g$), (type II); the latter is commonly considered as the main mechanism responsible for cell damage [5]. Both reactions can occur simultaneously and the ratio between the two processes depends on the PS, the substrate and the type of medium [5]. Because of the short lifetime (<0.04 μs) and radius of action (<0.02 μm) of reactive oxygen species, the damage site is restricted to the area in which reactive oxygen species are produced [6,7]. Therefore, an important advantage of PDT is that it provides a selective therapeutic effect, sparing surrounding normal tissue by preferential accumulation of the PS in tumor tissue and laser irradiation restricted to the target tissue.

The first-generation PS, porfimer sodium (marketed as Photofrin®, a complex mixture obtained by partial purification of hematoporphyrin derivative), used clinically to treat a number of solid tumors, including cancers of the lung, stomach, oesophagus and uterine cervix has been extensively investigated [8]. However, the use of porfimer sodium has several disadvantages: it is not a chemically

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defined entity, but rather a mixture of porphyrin monomers, dimers and oligomers; it causes prolonged skin photosensitization, lasting up to 4–6 weeks after treatment; in addition, the activating wavelength (630 nm) corresponds to a low absorption PS profile, and exhibits poor tissue penetration. In fact, one of the characteristics of the "ideal" PS for in vivo applications is a maximum light absorption in the red region of the visible spectrum (650–780 nm), in order to avoid the absorption of the light due to the presence of endogenous pigments, mainly haemoglobin, which strongly absorb light between 500 and 600 nm. On the other hand, wavelengths longer than 800 nm are energetically insufficient to produce O_2 ($^1\Delta_g$) and therefore are not used for PDT.

These considerations prompted an active search for novel, chemically well-defined PSs with improved biological properties, generally indicated as "second-generation photosensitizers". At present, it is known that cellular uptake and targeting of subcellular organelles occur more efficiently with lipophilic PSs, due to better diffusion through cell membranes [9–11]. Thus, the majority of the so-called "second-generation photosensitizers" are hydrophobic in nature, although excessive lipophilicity is often detrimental to PS efficacy because of the formation of aggregates in aqueous solution. Phthalocyanines are PSs which associate a highly lipophilic character with light absorbance in the red region of the visible spectrum, up to the far IR (600–800 nm). Phthalocyanine hydrophilicity can be modulated by the insertion of suitable substituents covalently linked at the periphery of the molecule [12–14] or axially bonded on a metal atom coordinated in the centre of the macrocycle [15,16]; in addition, phthalocyanines are known to exhibit a long lifetime in the triplet excited state, resulting in a highly efficient $O_2(^1\Delta_g)$ production [5,17].

In the present article, we report the design, synthesis and photodynamic activity of a panel of eight new zinc(II)-phthalocyanines. These PSs are characterized by the presence of ionic and polar substituents to modulate their amphiphilic character. The symmetrically structured derivatives **1**–**4** were obtained by a total synthesis, starting from the desired phthalonitrile or from 4-sulphophthalic acid. The non-symmetric phthalocyanines **5**–**8** were synthesized via chloromethylation of the commercial zinc(II)-phthalocyanine and subsequent substitution of the chlorine atoms with different nucleophiles.

All the newly synthesized compounds were tested for photodynamic cytotoxicity in vitro on a human colon adenocarcinoma cell line (HCT116) and the results were compared with those obtained with porfimer sodium.

2. Experimental

2.1. Physical measurements

UV–Vis absorption spectra were measured on a Perkin–Elmer Lambda 10 instrument. Mass spectrometric measurements were performed on a Thermo LCQ-MS instrument (ESI source). Elemental analyses were performed on a ThermoQuest NA 2100, C, H, N analyzer, equipped with an electronic mass flow control and thermal conductivity detector. Analytical thin-layer chromatographies (TLC) were performed using Macherey-Nagel Polygram CEL 300 UV_{254} (pre-coated plastic sheets, 0.1 mm thick). Cellulose (20 μm, Aldrich) was used for column chromatography.

Zinc-(tetrasulphonylchloride)phthalocyanine [18] and zinc-(di-chloromethyl)phthalocyanine [19] were synthesized according to the methods reported in the literature.

2.2. Synthesis of the zinc-phthalocyanines

2.2.1. Synthesis of (2,9,16,23-tetranitrophthalocyaninato) zinc(II) (as a mixture of isomers) (1)

4-Nitrophthalonitrile (50 mg, 0.29 mmol) was mixed, under N_2, with 80 mg (0.36 mmol) of $Zn(OAc)_2$. The solid mixture was heated at 210–220 °C until a dark green color appeared. After cooling, the solid mixture was treated with 10 ml of DMF and the suspension was filtered to eliminate the insoluble material. The phthalocyanine **1** (39 mg, 71% yield) was isolated as pure product by filtration after the addition of water to the DMF solution. UV–Vis (MeOH): 342 ($\varepsilon = 48\,000$); 666 nm ($\varepsilon = 83\,300$). Anal. Calc. for $C_{32}H_{12}N_{12}O_8Zn$: C, 50.71; H, 1.60; N, 22.18. Found: C, 50.91; H, 1.69; N, 22.08%. MS: *m*/*z* 758 [M + 1].

2.2.2. Synthesis of [2,9,16,23-tetrakis(3-pyriyiloxy) phthalocyaninato]zinc(II) (as a mixture of isomers) (2)

4-(4-Pyridyloxy)-phthalonitrile (300 mg, 1.30 mmol) was mixed, under N_2, with 650 mg (2.90 mmol) of $Zn(OAc)_2$. The solid mixture was heated at 210–220 °C until a green color appeared. The desired compound was extracted in MeOH with soxhlet for 8 h. The pure product was isolated by concentration to dryness (**2**) 83 mg (24% yield). UV–Vis (MeOH): 340 nm ($\varepsilon = 60\,300$); 670 nm ($\varepsilon = 95\,300$). Anal. Calc. for $C_{52}H_{32}N_{12}O_4Zn$: C, 65.45; H, 3.38; N, 17.61. Found: C, 65.82; H, 3.40; N, 17.41%. MS: *m*/*z* 955 [M + 1].

2.2.3. Synthesis of [2,9,16,23-tetrakis (N-ethoxyethanolsulphonamido)phthalocyaninato]zinc(II) (as a mixture of isomers) (3)

2-(2-Aminoethoxy)ethanol (AEE) (0.2 ml, 1.2 mol) and 0.1 ml of Et_3N were added to a dichloromethane solution (15 ml) of zinc-(tetrasulphonylchloride)phthalocyanine (231 mg, 0.24 mmol). The solution was then refluxed for 12 h. The crude material was purified by two consecutive column chromatographies (cellulose, MeOH). (**3**) 80 mg (27% yield). UV–Vis (MeOH): 344 nm ($\varepsilon = 48\,300$); 668 nm ($\varepsilon = 83\,200$);. Anal. Calc. for $C_{48}H_{42}N_{12}O_{16}S_4Zn$: C, 46.62; H, 3.42; N, 13.59. Found: C, 46.09; H, 3.40; N, 13.48%. MS: *m*/*z* 1237 [M + 1].

2.2.4. Synthesis of [2,9,16,23-tetrakis(N-(4-cyanophenyl)sulphonamido)phthalocyaninato]zinc(II) (as a mixture of isomers) (4)

4-Aminobenzonitrile (236 mg, 2 mmol) was added to a solution of 490 mg (0.5 mmol) of zinc-(tetrasulphonylchloride)phthalocyanine in 15 ml of CH_2Cl_2 and 0.1 ml of Et_3N and the solution was refluxed for 12 h. After this period, the desired compound was recovered as pure product, following two column chromatographies (cellulose, CH_2Cl_2:MeOH 8:2 then CH_2Cl_2:MeOH 2:8). (**4**) 53 mg (8.3% yield). UV–Vis (MeOH): 340 nm ($\varepsilon = 33\,300$); 604 nm ($\varepsilon = 12\,800$); 670 nm ($\varepsilon = 52\,800$). IR (cm^{-1}): 3409.7 ν(NH), 2226.5 ν(C≡N). Anal. Calc. for $C_{60}H_{32}N_{16}O_8S_4Zn$: C, 55.49; H, 2.48; N, 17.26. Found: C, 55.13; H, 2.40; N, 17.05%. MS: m/z 1300 [M + 1].

2.2.5. Synthesis of [2,9(16)-di(methylen(4-hydroxypyridinium))phthalocyaninato]zinc(II) chloride (as a mixture of isomers) (5)

4-Hydroxypyridine (345 mg, 3.63 mmol) was added to a solution of 262 mg (0.39 mmol) of zinc-(di-chloromethyl)-phthalocyanine in 5 ml of DMF and the solution was refluxed for 6 h. The product was precipitated by addition of a mixture of CH_2Cl_2:hexane 1:1 and recovered by filtration; the solid product was then washed with acetone to yield the pure **5** (212 mg , 62.9% yield). UV–Vis (MeOH): 340 nm ($\varepsilon = 64\,700$); 666 nm ($\varepsilon = 155\,500$). Anal. Calc. for $C_{44}H_{28}N_{10}O_2Cl_4Zn$: C, 61.09; H, 3.26; N, 16.19. Found: C, 61.22; H, 3.35; N, 16.00%. MS: m/z 866 [M + 1].

2.2.6. Synthesis of [2,9(16)-di(methylen(3-hydroxypyridinium))phthalocyaninato]zinc(II) chloride (as a mixture of isomers) (6)

3-Hydroxypyridine (526 mg 5.54 mmol) was added to a solution of 200 mg (0.30 mmol) of zinc-(di-chloromethyl)-phthalocyanine in 5 ml of DMF and the solution was refluxed for 6 h. The pure product was precipitated by addition of a mixture of CH_2Cl_2:hexane 1:1 and recovered by filtration. The solid product was then washed with acetone, yielding pure **6** (82 mg, 32% yield). UV–Vis (MeOH): 334 nm ($\varepsilon = 37\,500$); 668 nm ($\varepsilon = 82\,200$). Anal. Calc. for $C_{44}H_{28}N_{10}O_2Cl_4Zn$: C, 61.09; H, 3.26; N, 16.19. Found: C, 60.89; H, 3.24; N, 15.97%. MS: m/z 866 [M + 1].

2.2.7. Synthesis of [2,9(16)-di(methylen(3,4,5-trimethoxybenzyloxy))phthalocyaninato]zinc(II) (as a mixture of isomers) (7)

A DMF solution (5 ml) of trimethoxybenzyl alcohol (618 mg, 3.12 mmol) [20] was treated with 385 mg (3.42 mmol) of ^{t}BuOK. The solution was kept at RT for 30 min, then 300 mg (0.44 mmol) of zinc-(di-chloromethyl)-phthalocyanine were added. The solution was refluxed for 6 h then the desired product was precipitated by the addition of a mixture of CH_2Cl_2:hexane 1:1 and recovered pure after filtration. (**7**) 64 mg (15% yield). UV–Vis (MeOH): 340 nm ($\varepsilon = 74\,800$); 668 nm ($\varepsilon = 111\,600$). Anal. Calc. for $C_{60}H_{32}N_{16}O_8S_4Zn$: C, 64.37; H, 4.15; N, 11.55. Found: C, 63.99; H, 4.03; N, 11.59%. MS: m/z 971 [M + 1].

2.2.8. Synthesis of [2,9(16)-di(methylen(3-pyridiloxy-N-oxide))phthalocyaninato]zinc(II) (as a mixture of isomers) (8)

621 mg (5.54 mmol) of ^{t}BuOK were added to a solution of 307 mg (2.77 mmol) of 3-hydroxypyridine-*N*-oxide in 8 ml of DMF. The mixture was kept at RT for 30 min, then 200 mg (0.30 mmol) of zinc-(di-chloromethyl)phthalocyanine were added and the solution refluxed for 16 h. The pure product was precipitated after cooling, by the addition of a mixture of CH_2Cl_2:hexane 1:1 and recovered by filtration. The crude solid product was crystallized from hot MeOH and precipitated on cooling by the addition of Et_2O. (**8**) 123 mg (50.4% yield). UV–Vis (MeOH): 344 nm ($\varepsilon = 42\,300$); 676 nm ($\varepsilon = 72\,700$). IR (cm^{-1}): 1604.6 ν(C=C), 1125.3 ν(N–O). Anal. Calc. for $C_{44}H_{26}N_{10}O_4Zn$: C, 64.13; H, 3.18; N, 17.00. Found: C, 64.43; H, 3.30; N, 17.17%. MS: m/z 825 [M + 1].

2.3. Photobleaching measurements

Stock DMSO solutions of the Zn-Pcs, at the approximate concentration of 1×10^{-3} M, were diluted to 15 ml with 0.1 M phosphate-buffered saline (PBS) to obtain phthalocyanine concentrations of 5×10^{-5} M and a DMSO concentration <2%. Each solution was exposed to a 500 W tungsten-halogen lamp for 2 h, keeping the temperature at 37 °C with an aqueous filter. After 15 min a first 0.4 ml sample was withdrawn, diluted with 1.6 ml of PBS, and analyzed by means of UV–Vis spectroscopy. Photobleaching was determined evaluating the time dependent decrease of the intensity of the maximum absorption at about 668 nm, collecting a sample every 15 min.

2.4. Cytotoxicity studies

Human adenocarcinoma HCT116 cells, obtained from the American Type Culture Collection (Rockville, MD, USA) were maintained in DMEM (Mascia-Brunelli, Milano, Italy) supplemented with 1% glutamine, 1% antibiotic mixture and 10% fetal bovine serum (Mascia-Brunelli) at 37 °C in a humidified 5% CO_2 atmosphere, and were used when in *log*-phase growth. The antiproliferative effect of the different phthalocyanines (PSs) was assessed using the MTT assay [21]. Briefly, 5×10^4 cells/ml were seeded onto 96-well plates and allowed to grow for 48 h prior to treatment with different PS concentrations. As phthalocyanines **1**–**4**, **7** and **8** are insoluble in PBS, for each compounds a 1×10^{-3} M stock solution was prepared in DMSO and subsequently diluted in culture medium to the desired concentrations; in order to avoid vehicle-induced cytotoxicity, the final DMSO concentration in the culture medium never exceeded 2% (v/v). After 24 h, the PS-containing medium was replaced by PBS, and cells were irradiated under visible light (tungsten-halogen lamp

500 W) for 2 h (light irradiance 22 mW/cm^2 calculated as average value between 380 and 780 nm and determined with a LICOR-1800 spectroradiometer; light dose of 158.4 J/cm^2, considering the 2 h period of irradiation). At the end of this time, cells were incubated for 24 h at 37 °C in drug-free medium; MTT was then added to each well (final concentration 0.4 mg/ml) for 3 h at 37 °C and formazan crystals formed through MTT metabolism by viable cells were dissolved in DMSO. Optical densities were measured at 570 nm using a Universal Microplate Reader EL800 (Bio-Tek Instruments). IC_{50} values (i.e. PS concentrations affecting 50% of the cells) were estimated by non-linear regression analysis, using the GraphPad PRISM 3.03 software (GraphPad Software Inc., San Diego CA).

Possible intrinsic (i.e. non-photodynamic) cytotoxic effects of the PS were assessed on control cells treated as described above, but omitting the irradiation step, with PS concentrations up to 10-fold higher than those used for PDT experiments.

Flow cytometric analysis of the percentage of apoptotic cells was performed following 24 h exposure to PSs (5–20 ng/ml), 2 h irradiation and 24 h incubation in drug-free medium. Cells (5×10^5/sample) were detached with trypsin/EDTA, washed in PBS and fixed in ice-cold 70% ethanol for 20 min at −20 °C. After a further wash in PBS, cellular DNA was stained with 50 μg/ml propidium iodide in PBS in the presence of RNAse A (30 U/ml) at 37 °C for 30 min; the cell suspension was analysed on a Becton Dickinson FACScalibur instrument equipped with a 15 mW, 488 nm, air-cooled argon laser. The percentage of apoptotic cells in each sample was determined based on the sub-G1 peaks detected in monoparametric histograms; data were analyzed using Cell Quest software (Becton Dickinson).

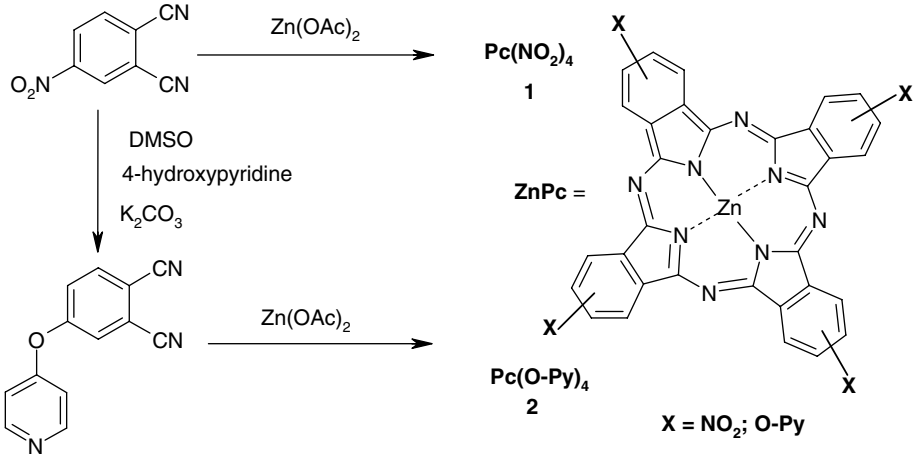
3. Results and discussion

A panel of zinc(II)-phthalocyanines was synthesized, based on the assumption that the presence of different substituents on 2–4 over the 16 possible external positions of the phthalocyanine frame would affect the hydrophobic/hydrophilic character of these compounds. This feature is crucial for both cell penetration and subcellular localization, as it is generally recognised that amphiphilic molecules, bearing both hydrophobic and hydrophilic moieties, exhibit improved tumor-selective uptake and retention, particularly when polar and non-polar groups are non-symmetrically distributed on the PS structures [22].

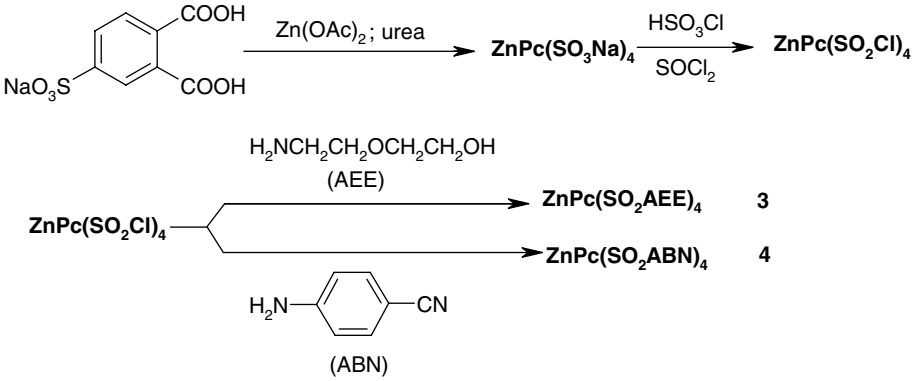
3.1. Synthesis

Phthalocyanines bearing substituents on the benzo units, can be directly obtained either via tetracyclization of substituted phthalonitriles or by the insertion of functional groups following phthalocyanine formation. In the former case, the synthesis leads to derivatives with one functional group on each of the four aromatic rings, whereas a variable degree of substitution will be obtained in the latter case, the number of the substituents being determined by the reaction conditions. Both syntheses result in formation of non-homogeneous products, because of the possible formation of numerous positional isomers. Separation of the regio-isomers of these compounds has yet to be addressed on a preparative scale, as it is reasonable to assume that differences in chemical and physical properties among isomers will be negligible.

Zinc(II)-phthalocyanines (**1**) and (**2**) were synthesized from the corresponding phthalonitrile. In the case of **2**, the precursor 4-[4-pyridyloxy]-phthalonitrile was prepared by a nucleophilic *ipso*-nitro substitution reaction carried out on of 4-nitrophthalonitrile with 4-hydroxypyridine in the presence of K_2CO_3 [23]. The product was isolated in a 22.5% yield, after crystallization. The cyclotetramerization of both 4-nitrophthalonitrile and 4-(4-piridyloxy)phthalonitrile was performed without solvent means of direct heating of the solid mixture of reagents in the presence of zinc(II) acetate. The zinc(II)-phthalocyanines (**1**) and (**2**) were isolated in 71% and 24% yield, respectively after purification (Scheme 1).



Scheme 1. Synthesis of zinc(II) phthalocyanines (**1**) and (**2**) from 4-nitrophthalonitrile.

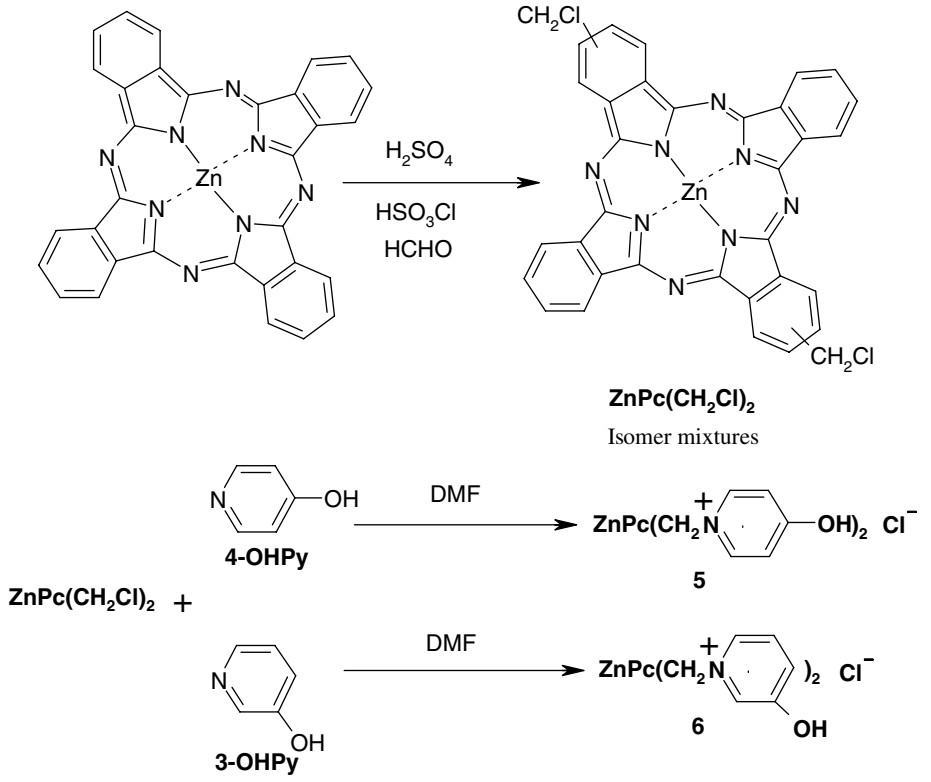


Scheme 2. Synthesis of zinc(II)-tetrasulphonylchloride phthalocyanine and subsequent reaction with amines yielding sulphonamido zinc(II)-phthalocyanines (**3**) and (**4**).

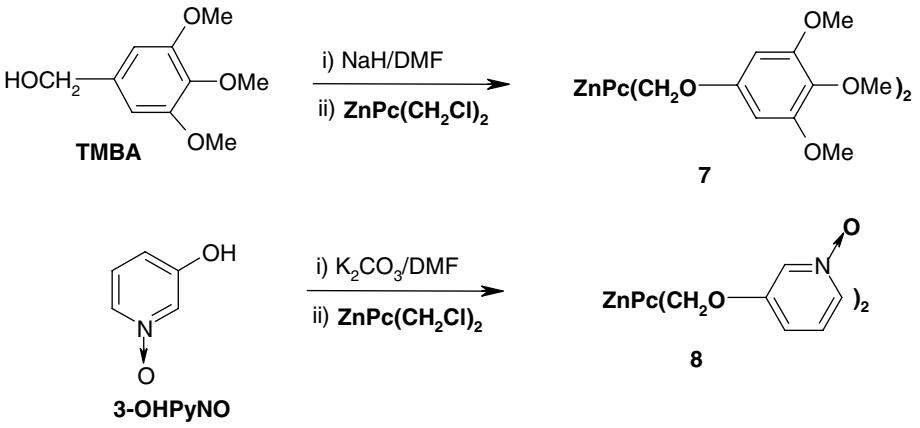
Zinc(II) phthalocyanines (**3**) and (**4**) were synthesized by reacting zinc(II)-(tetrasulphonyl chloride)phthalocyanine with two different amines producing four sulphonamido lateral chains; these substituents have been introduced with the aim to improve the solubility in water of these PSs.

The synthesis required the reaction of a Pc tetrasulphonylchloride derivatives, dissolved in CH_2Cl_2, with commercially available 2-(2-aminoethoxy)ethanol and 4-aminobenzonitrile, affording **3** and **4** in 27% and 8.3% yield, respectively (Scheme 2). In particular, the presence of four aminoethoxyethanol tails should increase the hydrophilicity of compound **3**.

The synthesis of the zinc(II)-phthalocyanines (**5**)–(**8**) set out from zinc-phthalocyanine featuring two chloromethyl groups, which are suitable for further synthetic modifications. Phthalocyanine chloromethylation was carried out dissolving zinc(II)-phthalocyanine in a sulphuric acid–chlorosulphonic acid mixture then generating the alkylating agent by addition of paraformaldehyde and sodium chloride as described by Griffiths et al. [19]. After 12 h at 80 °C, a mixture of isomers was obtained, mainly consisting of di-chloromethyl-phthalocyanine. From this compound, the introduction of further functional groups on the periphery of the phthalocyanines is straightforward. Cationic phthalocyanines **5** and **6** were obtained by reacting the chloromethyl derivative with 4-hydroxypyridine and 3-hydroxypyiridine, with 62.9% and 32% yields, respectively (Scheme 3).



Scheme 3. Synthesis of zinc(II)-dichloromethyl phthalocyanine and subsequent reaction with hydroxy-pyridines yielding dicationic zinc(II)-phthalocyanines (**5**) and (**6**).



Scheme 4. Synthesis of zinc(II)-phthalocyanines (**7**) and (**8**) via nucleophilic substitution of chlorine atoms of zinc(II)-dichloromethyl phthalocyanine with alcohols.

Non-ionic zinc(II)-phthalocyanines (**7**) and (**8**) were synthesized by reacting the di-chloromethyl precursor described above with potassium salts of trimethoxybenzyl alcohol and of 3-hydroxypyridine N-oxide, respectively (Scheme 4).

3.2. Chemico-physical properties

The absorbance in the visible region of the UV–Vis spectrum of the compounds here reported show a different profile depending either on the molecular structure and on the solvent used. As an example we here report the spectra of the non-ionic Pc **7** and those of the cationic Pc **5**, registered as DMSO or MeOH 10^{-5} M solutions (Fig. 1). Compound **7** gives the typical shape of aggregates in the former solvent (wide and unresolved bands in the 600–700 nm range with an intensity lower than that one of the Soret band), while the MeOH solution features the absorbance at 668 which is typical for a monomeric compound, although the absorbance due to the aggregates is still noticeable as shoulder of

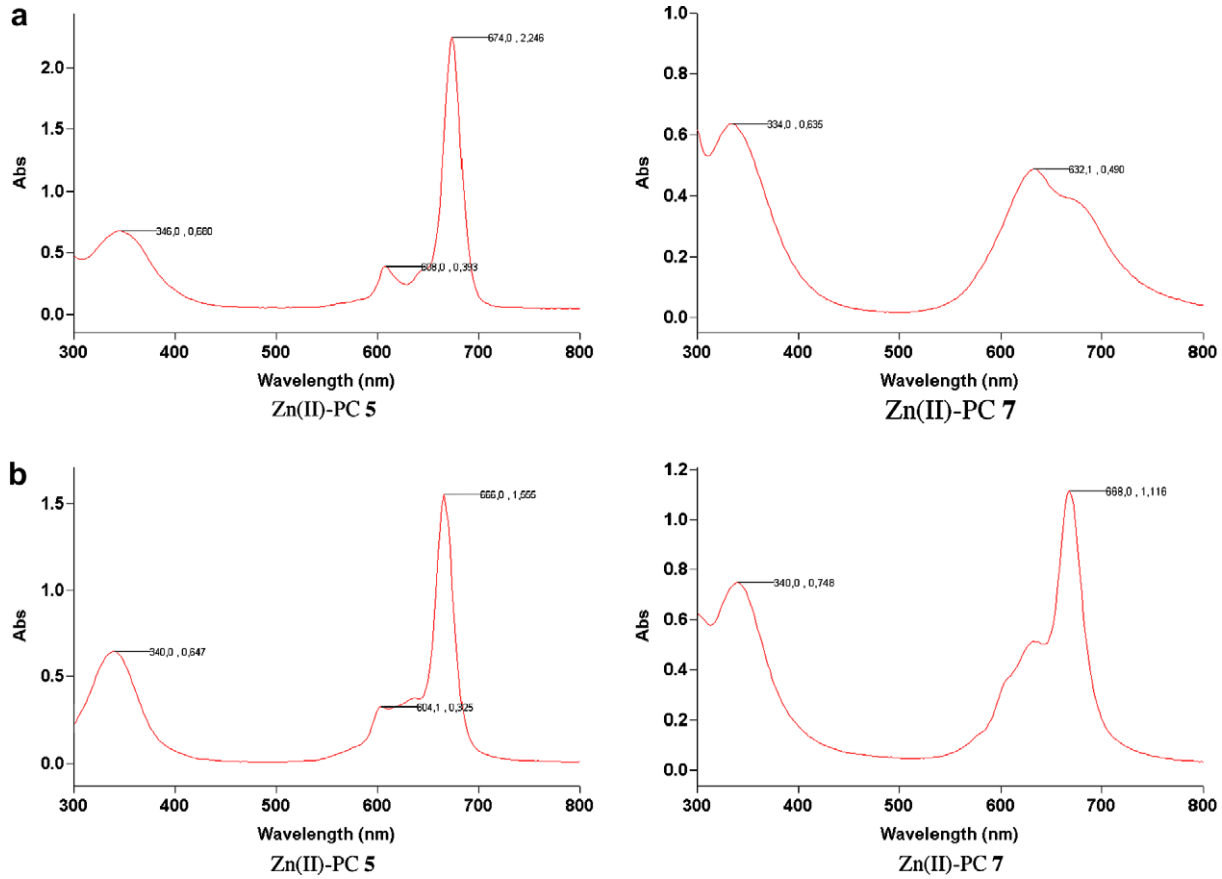


Fig. 1. Absorbance spectra in the visible region of 10^{-5} M solutions of zinc(II)-phthalocyanines (**5**) and (**7**) in DMSO (a) and in MeOH (b).

the main pick. On the contrary the dicationic photosensitizer **5** shows the characteristic intense absorbance at 674 and 666 nm in DMSO and MeOH, respectively. The 8 nm difference in the values of the wavelength of the maximum absorbance accounts for the difference in refractive index (n_D) of the two medium, as reported by Nyokong and co-workers [24].

Phthalocyanines, similarly to PSs belonging to the tetrapyrrolic family, are known to undergo partial demolition in the presence of oxidizing species; it is generally believed that bleaching can be mediated by photo-generated singlet oxygen [25], although some experimental details indicate that free radical-mediated photodegradation (Type-I) predominates [26]. In order to select an appropriate irradiation time for cytotoxicity studies, we first assessed the degree of photo-degradation for the newly synthesized compounds by following the decrease in intensity of the Q-band of the absorption spectrum. For all the Zn(II)-phthalocyanines tested, approximately 60% of the intensity of the initial Q-band was retained at the end of 2 h exposure to a 500 W tungsten-halogen lamp. The stability of porfimer sodium, which has been used as reference PS in cytotoxicity studies, was also assessed under the same conditions; porfimer sodium showed an extent of photobleaching comparable to that observed for phthalocyanines.

The observation of the absorbance decrease with the irradiation time only allows the detection of the demolition of the PSs chromophoric structure then, as the degradation rates are similar for all the investigated compounds, this process is apparently unaffected by the presence of different side arms or groups. A more reliable analysis would require the quantitative and qualitative determination of the structure of the demolition products for each PS; however, such detailed analysis is far too cumbersome to be performed routinely, and it is generally addressed when a particularly active novel compound is about to undergo clinical trials.

It may be worth emphasizing that the phthalocyanine concentrations used in photobleaching experiments (5×10^{-5} M) were significantly higher than those used for PDT assays on cell cultures (nanomolar range); thus, it is reasonable to hypothesize that, in the phthalocyanine concentration range actually used for cytotoxicity studies, the extent of photobleaching may be lower than that observed in photodegradation studies.

3.3. Cytotoxicity assays

The photodynamic activities of the phthalocyanines **1**–**8** were compared in in vitro assays on human colon adenocarcinoma cells (HCT116).

The IC_{50} values from dose/response curves obtained in HCT116 cells, following exposure to variable amounts to the different zinc(II)-phthalocyanines for 24 h and irradiation with visible light for 2 h, are reported in Table 1; porfimer sodium, the first drug receiving approval for the treatment of cancer [1], was also included as reference compound. The intrinsic cytotoxicity of PSs was assessed by omitting the irradiation step from the treatment protocol, and was found to be negligible in all cases, up to zinc(II)-phthalocyanine concentrations 10-fold higher than those used for PDT experiments (data not shown).

Table 1
IC_{50} values (mean ± SE) obtained from 4 to 6 independent experiments

Molecule	IC_{50} ng/ml ± SE	IC_{50} nM ± SE
Porfimer sodium	73.67 ± 8.04	–
Zn-Pc **1**	>1000	>1319
Zn-Pc **2**	>1000	>982
Zn-Pc **3**	>1000	>809
Zn-Pc **4**	>1000	>770
Zn-Pc **5**	18.20 ± 5.78[a]	21.04 ± 6.68
Zn-Pc **6**	90.00 ± 12.05	104.05 ± 13.93
Zn-Pc **7**	151.30 ± 46.10[a]	155.90 ± 47.51
Zn-Pc **8**	>1000	>1213

Statistically significant differences were assessed by the analysis of variance, followed by Dunnet's test.
[a] $p < 0.05$ vs. porfimer sodium.

Among the phthalocyanines tested, only the dicationic compound **5** was found to be significantly more phototoxic than porfimer sodium (IC_{50} 18.20 ± 5.78 vs. 73.67 ± 8.04 ng/ml; concentrations are expressed in ng/ml, because molecular weight cannot be calculated for porfimer sodium, which is an undefined mixture of oligomers deriving from hematoporphyrin).

The other cationic phthalocyanine in the panel, compound **6**, only showed a slightly difference in activity as compared to porfimer sodium (IC_{50} value for compound **6** 90.00 ± 12.05 ng/ml). It is interesting to note that compounds **5** and **6** only differ in the position of the OH group on the pyridyl moieties, yet they exhibit different phototoxic activities. This result is not totally unexpected as different effect of regio-isomers was already observed by our group, concerning phototoxicity of tetra- and diarylporphyrins; namely that the presence of the same substituent on a *meta* or on *para* position of the *meso*-phenyl rings results in a different phototoxicity [27].

The highly lipophilic Zn-Pcs (**1**)–(**4**) and (**8**) were practically devoid of phototoxicity, with IC_{50} values >1 μg/ml (not determined). As compounds **1**–**8** belong to the same class of PSs, these striking differences in the photoactivity of Pcs (**5**)–(**7**) with respect to that of Pcs (**1**)–(**4**), (**8**) must be related to some peculiar behavior in cell culture medium or inside the cells. As reported above, the absorbance profile of cationic and non-ionic compounds are quite different, the latter showing an UV–Vis typically related to the presence of aggregates, even in organic solvent such as DMSO and MeOH. Hence, it is convincible that the low efficacy of lipophilic photosensitizers is related to their poor solubility in aqueous solution, where aggregation phenomena occur, thereby reducing PS availability for cellular interactions (even though aggregation is expected to occur in lower extent at the nM concentrations used for the in vitro photoactivity investigations). On the other hand it is known that the balance between the hydrophilicity and the lipophilicity is particularly important for the

PSs' efficacy, since the highly polar compounds are easily administered in aqueous medium but hardly penetrate the lipophylic membrane of eukaryotic cells, on the other hand the highly lipophylic drugs would easily penetrate the membrane however they suffer of a difficult availability in water, then particular way for they administration should be adopted (i.e. liposomal formulation). In this context the phototoxicity of non-ionic zinc(II)-phthalocyanine (**7**), which was found active in a concentration range similar with that of porfimer sodium (IC_{50} 151.30 ± 46.10 ng/ml), can be explained. This compound features twelve methoxy groups that, behaving as a low weight polyethylene glycol, increase the hydrophilic character of the phthalocyanine. We have already reported a similar effect for PSs belonging to the tetraaryl-porphyrin class, where the dodecamethoxy compounds were found to be largely more active than the tetramethoxy derivatives, showing an activity comparable with those of porphyrins bearing four hydroxyl groups on their skeleton [28].

For the most active compound **5**, flow cytometric analysis of apoptotic cells was also performed, following exposure of the cells to the PS (5 – 10–20 ng/ml) for 24 h and subsequent irradiation for 2 h; again, porfimer sodium was used as reference PS. The results of these experiments (Fig. 2) show a significant difference between these two PSs, and indicate that apoptosis is a major mode of cell death induced by PDT with compound **5**; as the mode of cell death (apoptosis vs. necrosis) following photodynamic treatment largely depends on the subcellular localization of the PS, this suggests a different intracellular distribution for zinc(II)-phthalocyanine (**5**) and porfimer sodium.

In conclusion, we have presented the synthesis and in vitro photodynamic activity of a small panel of PSs belonging to the class of the Zn-phthalocyanines; among the tested compounds, Pc **5** proved to be particularly promising, as it exhibited a significant degree of cytotoxicity against human colon adenocarcinoma cells, mainly due to induction of apoptotic cell death. Furthermore, even though some of the molecules tested were poorly active, they could still serve as stepping stones towards the synthesis of novel PSs, as the presence of reactive functional groups (e.g. the hydroxyl groups on each side arm of compound **3**) allows the connection of a number of other substituents, thus producing a further modification of the polar character of these phthalocyanines, possibly yielding more active structures.

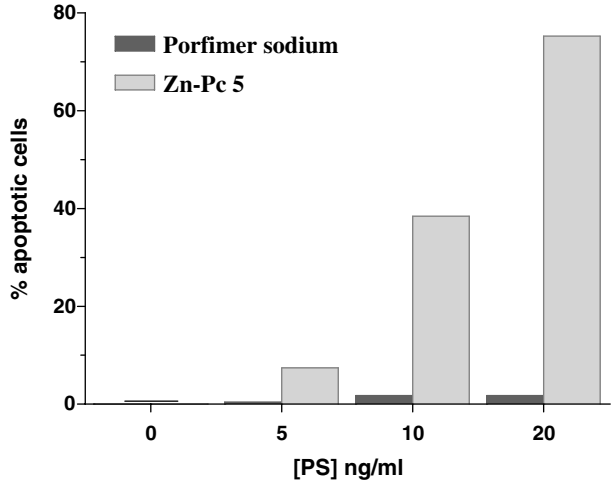


Fig. 2. Induction of apoptosis in HCT116 cells following photodynamic treatment with zinc(II)-phthalocyanin (**5**) and porfimer sodium (5, 10 and 20 ng/ml for 24 h, followed by 2 h irradiation and 24 h in PS-free medium).

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