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Photodynamic effects of porphyrin and chlorin photosensitizers in human colon adenocarcinoma cells

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Abstract—Photodynamic therapy (PDT) is a cancer treatment involving systemic administration of a tumor-localizing photosensitizer; this, when activated by the appropriate wavelength of light, interacts with molecular oxygen to form a toxic, short-lived species known as singlet oxygen, which is thought to mediate cellular death. Photofrin®, a complex mixture of porphyrin oligomers has recently received FDA approval for the photodynamic treatment of esophageal and endobronchial carcinoma, but its photodynamic and toxicity profiles are far from ideal. In the present study we evaluated a series of porphyrin-based PSs, some of which newly synthesized by our group, with the aim to identify agents with more favorable characteristics. For the most effective compounds in the porphyrin series, chlorin analogs were also synthesized; for comparison, the screening also included Photofrin®. Cytotoxicity studies were performed by the MTT assay on a cultured human colon adenocarcinoma cell line (HCT116); the results indicate that the 3,4,5-trimethoxyphenyl, 3OH- and 4OH-phenyl, and the sulfonamidophenyl derivatives are significantly more potent than Photofrin®. Flow cytometric studies and fluorescence microscopy indicate that in PDT-treated HCT116 cells death occurs mainly by apoptosis.

In summary, novel PSs described in the present study, belonging both to the porphyrin and chlorin series, have proven more effective than Photofrin® in killing colon cancer cells in vitro; extending these observation to in vivo models, particularly regarding the deeper reaching chlorin derivatives, might lead to significant advances in the development of tumor PDT.

1. Introduction

Photodynamic therapy (PDT) provides a viable therapeutic option for the treatment of a number of solid malignancies, as well as nonmalignant diseases. Systemic administration of a photosensitizing agent (PS), exogenously administrated or endogenously generated, is followed by irradiation with visible light of appropriate wavelength (i.e., compatible with absorption spectrum of the PS) and dose. Upon energy absorption, the PS is driven into an excited triplet state, which interacts with ground state molecular oxygen to produce singlet oxygen, a highly cytotoxic species with short lifetime (<0.04 μs) and radius of action (<0.02 μm).[1] It appears that both direct cytotoxic activity and microvascular damage concur to the therapeutic effects of PSs;[2] photodamage at specific subcellular sites, most notably mitochondria, ultimately leads to cell death by apoptosis.[3]

The application of PDT to cancer treatment is particularly attractive, as it holds the potential for minimal side toxicities, due both to the preferential distribution and longer retention times of PSs in tumors as compared with normal tissues, and to the fact that light irradiation is limited to the tumor region. Furthermore, the use of PDT is not precluded by prior radiotherapy, chemotherapy or surgery.[4]

Porfimer sodium (Photofrin®), a complex of porphyrin oligomers, was the first PS to win approval by regulatory agencies in several countries and is now licensed for treatment of cancers in the esophagus, lung, stomach, cervix, and bladder. However, a number of problems related with the use of Photofrin®, such as extended skin photosensitivity and poor absorption of tissue-penetrating red light, have led to the development

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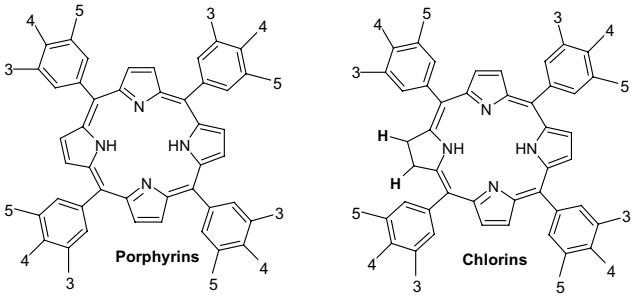
of novel photosensitizers with more favorable characteristics. These so-called second-generation PSs have shorter periods of photosensitization, longer activation wavelengths (and, therefore, are activated deeper within tissues), higher yields of singlet oxygen, and tumor selectivity. A large number of polyunsaturated compounds with different structures have been actively investigated, including chlorins, texaphyrins, purpurins, and phthalocyanines; however, only one new agent, the *m*-tetra(hydroxyphenyl)chlorin (*m*-THPC or temoporfin, marketed as Foscan®), has recently been approved by the European Medical Agency (EMEA) for use in head and neck squamous cell carcinomas.

The present study is a preliminary screen of the photodynamic activity of a small number of compounds obtained by modification of the tetraaryl porphyrin/chlorin ring (Fig. 1). The choice of the core structure was based on a number of considerations: (i) temoporfin, one of the most active PSs currently in clinical trials, is a tetraarylchlorin derivative; (ii) a variety of substituents, featuring different polarity and length, can be introduced on the *meso*-phenyls, either by using different substituted aromatic aldehydes for the synthesis or by chemical modification of the tetraphenyl-porphyrin skeleton, and this could yield novel PS with a better profile than the agents in current clinical use. A secondary goal in this study was to establish whether the presence of a tetraaryl porphyrin or tetraaryl chlorin ring significantly affects the photodynamic activity and cell penetration characteristics of these compounds. In vivo applications of porphyrin derivatives are hampered by their spectroscopic characteristics, which do not feature absorption in the red region (they exhibit an intense Soret absorption band around 420 nm and a lower band at 510 nm) and, therefore, are only activated by wavelengths with limited tissue penetration; however, the design and synthesis of tetraaryl porphyrins, as well as their in vitro use in tumor cells, are far less cumbersome as compared to the corresponding tetraaryl chlorin derivatives, and this makes them eminently suitable for in vitro screening of large panels of compounds. The most active compounds in the screen could then be converted to the corresponding chlorins (which exhibit an absorption band at 650 nm), in view of subsequent in vivo applications.



Porphyrins	3	4	5
1	OCH_3	H	H
2	H	OCH_3	H
3	OCH_3	OCH_3	OCH_3
4	H	SO_2AEE *	H
5	OH	H	H
6	H	OH	H
7	OH	OH	OH

Chlorins	3	4	5
9	H	OH	H
10	OCH_3	OCH_3	OCH_3
11	OH	OH	OH
12	H	SO_2AEE *	H

* SO_2AEE = $SO_2NHCH_2CH_2OCH_2CH_2OH$

Figure 1. Photosensitizer structures.

Table 1. IC_{50} values (± SE) obtained from 4–6 independent experiments. Statistically significant differences were assessed by the analysis of variance, followed by Dunnet's test, comparing PSs **1**–**12** versus Photofrin®

PS	IC_{50} (ng/mL) ± SE	IC_{50} (nM) ± SE
Photofrin®	73.67 ± 8.04	n.d
1	>10,000*	>13,608
2	4428 ± 125*	6026 ± 170
3	10.74 ± 1.82*	11 ± 1.86
4	23.20 ± 3.46*	18 ± 2.68
5	5.13 ± 0.98*	7.5 ± 1.4
6	3.07 ± 0.37*	4.52 ± 0.6
7	221.81 ± 36.42*	274 ± 44.99
8	24.8 ± 1.28*	36.4 ± 1.88
9	28.69 ± 4.12*	42 ± 6.05
10	6.51 ± 1.32*	6.66 ± 1.35
11	3358.00 ± 96*	4178 ± 120
12	17.74 ± 1.26*	13.8 ± 0.98

* $p < 0.05$ versus Photofrin®.

Twelve tetraaryl derivatives, including five porphyrin/chlorin pairs, were tested for photodynamic cytotoxicity on a human colon adenocarcinoma cell line (HCT116) and the results were compared with those obtained with Photofrin®. The series included a number of derivatives newly synthesized by our group and/or for the first time tested (Table 1, **3**,**4**, **7**, **10**, **11**, **12**), as well as temoporfin (**8**) and its tetraaryl porphyrin analog (**5**).

2. Results and discussion

The 'methoxy' porphyrins **1**–**3** were synthesized by condensation of aromatic aldehyde and pyrrole, following the general method described by Lindsey and co-workers, in 41%, 22%, and 33% yields, respectively, after column chromatography.[5] Compound **4** was isolated as previously reported,[6] while the 'hydroxy' porphyrins **5**–**7**, were obtained in good yields from the parent methoxy through reaction with BBr_3 in CH_2Cl_2.

The choice of the aryl substituents of the porphyrin rings was dictated by the necessity to modulate the hydrophilic/lipophilic character of the molecules, the starting point being tetraphenylporphyrin (H_2-TPP), which is the most lipophilic and is known as a poorly efficient photosensitizer. Although the exact mechanism of sensitizer uptake by tumor cells is still unknown, there is evidence that porphyrins are bound by plasma low density lipoproteins (LDLs) and internalized following interaction of LDLs with their receptors on cell membranes; binding to LDLs is probably related to the amphiphilic character of the molecule.[7] A minor change in lipophilicity can be obtained by the introduction of 1–3 methoxy appendix for each phenyl. In this series, compound **3**, featuring three methoxy groups on each phenyl can be considered as the analogous of the so-called 'pegylated' porphyrins, which have been studied as an alternative to the hydroxy substituted ones, and have shown an increased tumor selectivity and a prolonged activity in vivo.[8]

The methoxy groups have the advantage to easily undergo further transformation to the corresponding hydroxy derivatives by reaction with BBr_3 at 0 °C. The presence of one or more hydroxyl groups increases the degree of hydrophilicity of the porphyrins; in this respect, methoxyphenyl-porphyrins are considered the precursor of the hydroxyphenyl-containing tetrapyrrole macrocycles, of which the *m*-THCP is the most valuable example.

Chlorosulfonation of phenyl rings is a well known procedure, which allows the anchorage of side arms to the tetraaryl porphyrin structures; on the H_2-TPP it occurs on the four *para*-positions, yielding a symmetrically substituted chlorosulfonyl porphyrin.[9] The chlorosulfonic group can then be reacted with oxygen or nitrogen nucleophiles to give sulfonic ester or sulfonamide; we chose to synthesize the latter compound because of its higher stability versus hydrolysis. The aminoethoxyethanol (AEE) chain was then inserted with the aim to modify the lipophilic character of the tetraphenylporphyrin.

Chlorins **8**–**10** and **12** were synthesized from the corresponding porphyrin by reaction with toluene-4-sulfonylhydrazide, anhydrous K_2CO_3, and pyridine, using the method described by Whitlock et al.,[10] and were recovered in about 50%, 68%, and 92% yields, respectively. In some experiments the formation of bacteriochlorins (BCs) could not be avoided, as evidenced by the presence of an absorption band at 730 nm in UV–vis spectra; the product of porphyrin over-reduction was eliminated by carefully treating the mixture with a diluted toluene solution of chloranyl. Chlorin **11** could not be obtained from the corresponding 3,4,5-trihydroxy phenyl porphyrin; however, it was isolated from chlorin **10** after demethylation with BBr_3.

The formation of BCs as side products of chlorin synthesis deserves a comment. The reduction of porphyrins with tosylhydrazide and base is quite trivial and it is generally carried out with a large excess of reducing agent; chlorin concentration is spectroscopically evaluated by measuring the appearance of an absorption band at 650 nm. The ratio between the intensity of the Soret absorption band at 420 nm and the absorption band at 650 nm was assumed as an index of chlorin formation. In order to achieve the desired value (420/650 = 6) the reaction should be prolonged for many hours; therefore, these harsh conditions end in the unavoidable formation of some BCs, which is identified by the 730 nm absorption band. Actually, based on their spectroscopic characteristics, BCs could be even more interesting than chlorins for in vivo applications, but the advantage of activation by highly tissue-penetrating wavelengths is overthrown by their greater thermal and photochemical instability. In vivo experiments comparing *m*-THPC with the corresponding BC *m*-THPBC, indicate that the photobleaching rate is 20 times greater for the latter than for the chlorin derivative.[11]

2.1. Cytotoxicity studies

Figure 2 shows the dose/response curves obtained in HCT116 cells following exposure to the different porphyrin (panel A) and chlorin (panel B) derivatives for 24 h and irradiation with visible light for 2 h, and compares them with the curve obtained for Photofrin® under identical experimental conditions. The intrinsic cytotoxicity of tertraaryl derivatives was assessed by omitting the irradiation step from the treatment protocol, and was found to be negligible in all cases (data not shown). Thus, photoactivation is an absolute requirement for the cytotoxic activity of these

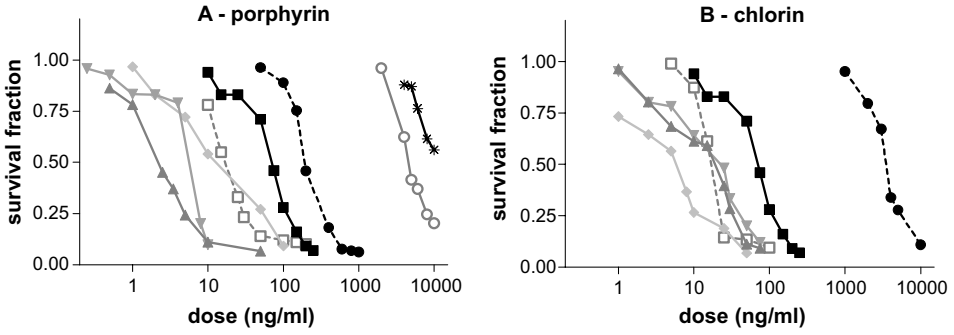


Figure 2. Representative dose–response curves obtained in HCT116 cells following exposure to the different porphyrin (panel A) and chlorin (panel B) derivatives for 24 h and irradiation with visible light for 2 h. Curves obtained with PSs 1-12 are compared with the curve obtained for Photofrin (■) under identical experimental conditions. Panel A: ✳ 1, ○ 2, ◆ 3, □ 4, ▼ 5, ▲ 6, ● 7; panel B: ▼ 8, ▲9, ◆ 10, ● 11, □ 12.

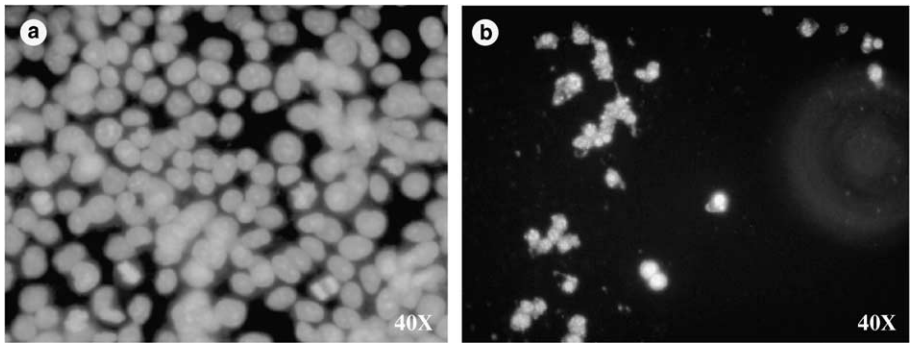


Figure 3. Visualization of apoptotic nuclei by fluorescent microscopy in HCT116 cells following exposure to PSs: panel A—control; panel B—photosensitizer 6 (4-hydroxy porphyrin) for 24 h (3 ng/mL) and irradiation with visible light for 2 h.

compounds. All the compounds tested proved to be significantly more cytotoxic than Photofrin®, except the trihydroxy-substituted derivatives (both porphyrin and chlorin) and the 4-methoxy- and 3-methoxy-porphyrin analogs, which were practically devoid of effect. This is confirmed by the IC_{50} values calculated for each curve and reported in Table 1. Interestingly, cell death induced by tetraaryl derivatives occurs mainly by apoptosis, in agreement with observations reported by other authors.[12,13] Apoptotic nuclei following photodynamic treatment of HCT116 cells with 4-OH-tetraaryl porphyrin (the most effective compound in the series tested) at a concentration corresponding to its IC_{50} value are shown in Figure 3, in which the chromatin condensation typical of the late phases of apoptosis is clearly visible.

Notably, the cytotoxic activity exerted by **3**, **4**, **5**, **6**, and the newly synthesized derivatives (**10**, **12**) was greater than that exhibited by temoporfin (**8**), while chlorin **9** showed comparable cytotoxic activity. Temoporfin has been approved in 2001 by the European Medical Agency (EMEA) for use in head and neck squamous cell carcinomas, and is the latest addition to the small group of compounds currently used for PDT. Its photodynamic and toxicity profiles compare favorably with those of Photofrin®: in fact, in the present study it was found to be significantly more cytotoxic than the latter (IC_{50} values 24.8 ± 1.28 ng/mL vs. 73.67 ± 8.04, $p < 0.05$; mean ± SE), and this results confirm previous in vitro and clinical observations.[14,15] In addition, the main-adverse effects of temoporfin have been related to local tumor necrosis, whereas extended photosensitivity, representing the major drawback to the clinical use of Photofrin®, can easily be prevented with the adoption of appropriate measures. Among the novel PSs, both trihydroxy derivatives (compounds **7** and **11**, IC_{50} values 221.81 ± 36.42 and 3358.00 ± 96.00 ng/mL, respectively; mean ± SE) display a significantly lower cytotoxicity than either temoporfin (compound **8**) or Photofrin® (IC_{50} values 24.8 ± 1.28 and 73.67 ± 8.04 ng/mL, respectively; mean ± SE); therefore, it is hard to foresee any further developments for these derivatives. The two aminoethoxyethanol derivatives (compounds **4** and **12**), for the first time used as photosensitizers, seem far more promising as lead compounds: in fact, while their cytotoxic effect does not significantly differ from that of temoporfin (IC_{50} values 23.20 ± 3.46 ng/mL for the porphyrin and 17.74 ± 1.26 ng/mL for the chlorin; mean ± SE), their molecules contain a polyfunctionalized side arm, to which a number of moieties could be conveniently attached to improve pharmacokinetics, cellular uptake, subcellular localization and/or tumor targeting. The branched arms can be modified at the sulfonyl chloride level, reacting nitrogen nucleophiles with different structures or, in a subsequent step, exploiting the presence of an hydroxyl moiety. As an example, α-aminoacid methylester residues have recently been grafted on the sulfonated tetraphenyl-porphyrin and the phototoxic activity was tested on HT29 adenocarcinoma cells.[16]

Both trimethoxy derivatives (**3** and **10**, IC_{50} values 10.74 ± 1.82 and 6.51 ± 1.32 ng/mL, respectively; mean ± SE) were found to be significantly more cytotoxic than either temoporfin (compound **8**) or Photofrin®. However, these derivatives contain 3,4,5-substituted phenyl rings and distinctions among the reactivities of the three positions are no longer possible; therefore, their molecular structures will not bear much further alterations, thus limiting future developments for these compounds. To the best of our knowledge, compounds **3** and **10** have not yet been tested on HCT 116 cells; about trimethoxy-phenyl porphyrin, Dolphin has recently reported the activity of dihydroxychlorin derivative of compound **3** on L1210 cells.[17]

The relative cytotoxicities of compounds containing a porphyrin or a chlorin rings deserve some comments. To the best of our knowledge, direct comparisons of the phototoxicity of porphyrins and the corresponding chlorins have never been reported in the literature, as most of the studies address differences in the absorption profiles between the two families rather than biological effects. The results obtained in the present study indicate that the presence of a fully unsaturated tetrapyrrole ring or a mono hydrogenated ring in the tetraaryl derivatives may significantly affect the cytotoxic properties of the compounds. Whereas no significant differences were observed for the trimethoxy (**3** and **10**) and aminoethoxyethanol (**4** and **12**) pairs, the 3-hydroxy and 4-hydroxy chlorin derivatives (**8** and **9**, IC_{50} values 24.8 ± 1.28 and 28.69 ± 4.12 ng/mL, respectively; mean ± SE) were significantly less cytotoxic than the corresponding porphyrins (**5**and **6**, IC_{50} values 5.13 ± 0.98 and

3.07 ± 0.37 ng/mL, respectively; mean ± SE). Preliminary uptake data obtained for some of the tested PSs indicate that photodynamic activity is loosely correlated to the intracellular PS levels and that, in the case of the 4-hydroxy porphyrin/chlorin pair, the chlorin derivative is less cell-permeant than the corresponding porphyrin, as assessed by fluorometric measures on cell extracts (data not shown). This observation can hardly be related to structural differences between porphyrins and chlorines; in fact, while it is obvious that the presence of one extra double bond can influence the spectroscopic behavior of the polyunsaturated structure, the whole conformation of the molecule is not affected by the addition of two hydrogen atoms. In conclusion, the observed difference of phototoxicity between porphyrins **5**, **6** and the corresponding chlorins **8**, **9** cannot be accounted for by their general structural features.

Another peculiar observation inferred from the results of the present work is that, under the conditions adopted for cytotoxicity assays, no substantial differences in activity were observed between 3- and 4-hydroxyphenyl substituted tetrapyrroles, either in the porphyrin or chlorin series. About chlorins, recent and old papers report an activity 1–2 order of magnitude higher for the *meta* isomer with respect to the *para*, in both in vitro and in vivo experiments.[17,18] These results cannot be accounted for by structural evidence, and, as far as we know, no explanation has been offered for the observed difference in activity. In a HPLC/MS study currently ongoing in our laboratory 3-hydroxy derivatives do exhibit a higher affinity for the stationary phase (RP-C18) than the 4-OH substituted agents; this observation indicates that 3-hydroxy-derivatives are more lipophilic, which could account for their greater phototoxicity. However, differences in cytotoxicity were not apparent in HCT116 cells, suggesting that cell type-specific factors may also play a role in the response to PDT.

In summary, we can conclude that trimethoxyphenyl derivatives (**3**, **10**) and hydroxyphenyl compounds (**5**, **6**, **8**, **9**) are the most active among the tested compounds. Substituting an aminoethoxyethanol moiety in the phenyl rings of porphyrins and chlorins yields compounds (**4**, **12**) with cytotoxic potencies very similar to those exhibited by currently used PS that lend themselves to further chemical modifications aimed at improving their antitumor efficacy and/or their pharmacokinetics. Subsequent in vitro assessments of the photodynamic activity of modified aminoethoxyethanol derivatives will have to be performed directly on chlorin derivatives, as they have better light absorbing characteristics than the corresponding porphyrins (and are, therefore, better candidates for in vivo applications) and may differ considerably from the latter in both cell permeability and cytotoxicity.

3. Experimental

3.1. General

UV–vis absorption spectra were measured on a Perkin–Elmer Lambda 10 instrument. ^{1}H NMR spectra were recorded on a Bruker 400 MHz spectrometer in $CDCl_3$ or [d_6] DMSO; chemical shifts are expressed in ppm relative to chloroform (7.26). Mass spectrometric measurements were performed on a Finnigan LCQ-MS instrument fitted with an electrospray ionization (ESI) or atmospheric pressure chemical ionization (APCI) ion sources, equipped with an ion trap mass analyzer. HPLC analyses were conducted with a Thermo Separation Products (TSP) instrument coupled with a Finnigan LCQ-MS. The instrument was fitted with a 0.21 × 15 cm column (Supelco, Discovery) packed with C-18 reversed-phase particles (5 μm) and operated with an isocratic elution with A:B/30:70 ratio (A = H_2O, 0.1% CH_3COOH; B = CH_3CN:CH_3OH/80:20) at 0.2 mL/min.

Elemental analyses were performed on a ThermoQuest NA 2100, C, H, N analyzer, equipped with an electronic mass flow control and a thermal conductivity detector.

Analytical thin-layer chromatography (TLC) was performed using Merck 60 F254 silica gel (precoated sheets, 0.2 mm thick) or on Macherey–Nagel F254 silica gel C_{18}-100 (precoated sheets, 0.25 mm thick). Silica gel 60 (70–230 mesh, Merck) was used for column chromatography.

Aromatic aldehydes, 5,10,15,20-tetraphenyl-21H,23H-porphyrin [H_2-TPP] and 5,10,15,20-tetra-(4-hydroxyphenyl)-21H,23H-porphyrin (**6**), were commercial products (Sigma–Aldrich) and used as received. Pyrrole and $BF_3{\cdot}Et_20$ were freshly distilled prior to use. Dichloromethane used for porphyrin synthesis was distilled from $CaCl_2$ directly into the reaction flask.

4. Synthesis of free base tetraarylporphyrins

5,10,15,20-Tetra(3-methoxyphenyl)-21H,23H-porphyrin [H_2-T(*m*-OMeP)P, **1**], 5,10,15,20-tetra(4-methoxyphenyl)-21H,23H-porphyrin [H_2-T(*p*-OMeP)P, **2**], and 5,10,15,20-tetra(4,5,6-trimethoxyphenyl)-21H,23H-porphyrin [H_2-T(OMe_3P)P, **3**] were synthesized via condensation of the corresponding aromatic aldehydes and pyrrole under mixed-acid catalysis, as recently reported by Lindsey and co-workers.[5] The chlorosulfonyl derivative of H_2-TPP was obtained by reacting free-base porphyrins with chlorosulfonic acid, following the method described by Rocha Gonsalves et al.[9] 5,10,15,20-tetra-(4-*N*-ethoxyethanolsulfonamidophenyl)-21H,23H-porphyrin [H_2-T(*p*-SO_2 AEE)P-P, **4**] was synthesized as previously described.[6]

The general procedure for porphyrin syntheses is fully described for the first compound, the others being prepared under similar conditions.

4.1. 5,10,15,20-Tetra(3-methoxyphenyl)-21H,23H-porphyrin [H_2-T(*m*-OMe)P-P, 1]

$BF_3{\cdot}Et_2O$ (6 μL, 6×10^{-3} mmol) and 0.35 mL (4.5 mmol) of trifluoroacetic acid (TFA) were added to a solution of 0.6 mL (0.681 g, 5 mmol) of *m*-anisaldehyde and 0.35 mL (0.345 g, 5 mmol) of freshly distilled pyrrole in 500 mL of CH_2Cl_2; the mixture was kept at rt for 2 h. When all the

aldehyde was reacted (TLC:SiO_2; hexane/CH_2Cl_2 6/4) 1.01 g (4 mmol) of chloranyl were added and the mixture was kept under reflux for 4 h. The solvent was evaporated and the raw material purified by column chromatography (SiO_2; CH_2Cl_2). The fractions were tested by TLC, those containing only one single spot were collected and the recovered product was further purified by refluxing with MeOH (20 mL). The solid matter was filtered and washed on the filter with a small amount of CH_2Cl_2 affording 374 mg (41%) as a purple solid; UV–vis$_{(dichloromethane)}$: 418 nm (ε = 189,000); 514 nm (ε = 12,000). ^{1}H NMR ($CDCl_3$) δ: −2.77 (s, 2H); 4.00 (s, 12H); 7.36 (dd, 4H); 7.67 (t, 4H); 7.83 (t, 8H); 8.91 (s, 8H). IR (KBr): 2930 cm^{-1} (ν CH); 1152 cm^{-1} (ν CO). HPLC: retention time = 4.49 min. MS-APCI$^+$: *m/z* 735.6 (100%); 736.5 (45%). Molecular weight calculated for $C_{48}H_{38}N_4O_4$ = 734.8.

4.2. 5,10,15,20-Tetra(4-methoxyphenyl)-21H,23H-porphyrin [H_2-T(*p*-OMe)P-P, 2]

Compound **2** was synthesized as described above; the isolated pure product was 315 mg, corresponding to 21.8% yields. UV–vis$_{(dichloromethane)}$: 422 nm (ε = 253,000); 518 nm (ε = 18,000). ^{1}H NMR ($CDCl_3$) δ: −2.73 (s, 2H, *NH*); 4.12 (s, 12H); 7.30 (d, 8H); 8.14 (d, 8H); 8.88 (s, 8H). IR (KBr): 2927 cm^{-1} (ν CH); 1147 cm^{-1} (ν CO). HPLC: retention time = 6.45 min. MS-APCI$^+$: *m/z* 735.5 (100%); 736.6 (45%); MS-APCI$^-$: *m/z* 734.4 (100%). Molecular weight calculated for $C_{48}H_{38}N_4O_4$ = 734.8.

4.3. 5,10,15,20-Tetra(3,4,5-trimethoxyphenyl)-21H,23H-porphyrin [H_2-T(OMe)$_3$P-P, 3]

3,4,5-Trimethoxybenzaldehyde (981 mg, 5 mmol) and 0.35 mL (5 mmol) of pyrrole were reacted as described for of compound **1**. The raw material was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH 98/2) and the recovered product was crystallized dissolving the solid into the minimum amount of dichloromethane then settling as a precipitate by careful addition of hexane (CH_2Cl_2/hexane 1/2; v/v) to give 400 mg (33%) of the pure product.

UV–vis$_{(water)}$: 430 nm (ε = 133,000); 518 nm (ε = 22,000). IR (KBr): 2924 cm^{-1} (ν CH); 1122 cm^{-1} (ν CO). ^{1}H NMR ($CDCl_3$) δ: −2.75 (s, 2H), 3.99 (s, 24H, OCH_3); 4.21 (s, 12H, OCH_3); 7.49 (s, 8H); 8.98 (s, 8H). HPLC: retention time = 2.64 min. MS-APCI$^+$: *m/z* 975.5 (100%); 976.5 (50%) 978 (5%). Molecular weight calculated for $C_{56}H_{54}N_4O_{12}$ = 975.0.

5. Synthesis of hydroxy substituted porphyrins from methoxy porphyrins

5.1. 5,10,15,20-Tetra(3-hydroxyphenyl)-21H,23H-porphyrin [H_2-T(*m*-OH)P-P, 5]

A solution of 100 mg (0.136 mmol) of porphyrin **1** in 14 mL of CH_2Cl_2 was stirred at 0 °C for 0.5 h, then 2.72 mL (2.72 mmol) of 1 M BBr_3 solution was added. The mixture was kept at 0 °C for 1 h and at rt for 18 h; after this period, 30 mL of CH_2Cl_2 was added to the reaction mixture together with the required amount of 1 M aqueous NaOH to neutralize the mixture. The layers were separated and the organic phase was further washed with water, dried (Na_2SO_4) and concentrated to dryness yielding a solid product (31 mg 33%). ^{1}H NMR ($CDCl_3$) δ: −2.97 (s, 2H, NH); 7.24 (d, 4H); 7.60 (dd, 12H); 8.89 (s, 8H); 9.89 (s, 4H, OH). HPLC: retention time = 2.57 min. UV–vis$_{(water)}$: 420 nm (ε = 102,000); 516 nm (ε = 11,000). IR (KBr): 3402 cm^{-1} (ν OH); 1605 cm^{-1} (ν C=C); 1259 cm^{-1} (ν CO). MS-ESI$^+$: *m/z* 679.7 (100%); 680.6 (44%); MS-ESI$^-$: *m/z* 677.4 (100%); 678.4 (40%). Molecular weight calculated for $C_{44}H_{30}N_4O_4$ = 678.7.

5.2. 5,10,15,20-Tetra(3,4,5-trihydroxyphenyl)-21H,23H-porphyrin [H_2-T(OH)$_3$P-P, 7]

A solution of 142 mg (0.146 mmol) of porphyrin in 15 mL of CH_2Cl_2 was treated with 0.6 mL (0.6 mmol) of BBr_3 solution as described above. The insoluble product was recovered by filtration and purified by gel permeation column chromatography on Bio-Beads resins, with CH_3CN as eluant, to give 73 mg (62%) of the pure product. UV–vis$_{(water)}$: 424 nm (ε = 101,000); 516 nm (ε = 8500). IR (KBr): 3512 cm^{-1} (ν OH). ^{1}H NMR (DMSO) δ: 7.62 (s, 8H); 8.60 (s, 8H); 9.80 (m, 12H, OH). MS-ESI$^+$ (flow injection) 807.5 highest peak of molecular cluster; MS-ESI$^-$ 805.3. Molecular weight calculated for $C_{44}H_{30}N_4O_{12}$ = 806.7. Anal. Calcd: C, 65.50; H, 3.74; N, 6.94. Found; C, 66.18; H, 3.77; N, 6.82.

6. Synthesis of chlorins from porphyrins

6.1. 5,10,15,20-Tetra(3-hydroxyphenyl)-2,3-dihydro-21H,23H-chlorin [H_2-T(*m*-OH)P-CL, 8]

Toluene-4-sulfonylhydrazide (87.8 mg, 0.472 mmol) and 163 mg (1.18 mmol) of K_2CO_3 were added to a solution of 80 mg (0.118 mmol) of porphyrins **5** in 10 mL of pyridine; the mixture was refluxed for 2 h; the reaction progress was monitored by means of UV–vis spectroscopy, based on the occurrence of the chlorin band at 650 nm. The same initial amount of hydrazide and K_2CO_3 were added every hour until the reaction was completed (determined evaluating the ratio of the intensity between the band at 420 nm and that one at 650 nm; this ratio must be equal or lower than 6). In some cases, the formation of an absorption band at 730 nm was detected, which is characteristic of the product of over-reduction (bacteriochlorin). At the end of the reaction, 35 mL of AcOEt and 18 mL of H_2O were added and the mixture was kept at 70 °C for 1 h. The organic layer was isolated, washed a few times with HCl 10%, then with H_2O, dried (Na_2SO_4) and concentrated to yield a solid product (38 mg 47.4%). The absorption spectra of the isolated compound was again controlled and, in the case of the presence of the peak at 730 nm, the solid was dissolved in CH_2Cl_2 and treated with a diluted toluene solution of chloranyl. This procedure must be carried out with particular attention to avoid oxidation of the chlorin back to the initial porphyrin.

As the final step of the reaction, the solution was concentrated and purified by column chromatography (SiO_2; CH_2Cl_2/MeOH 9/1) to give 30 mg (37%) of the pure product.

UV–vis$_{(water)}$: 420 nm (ε = 74,700); 650 nm (ε = 18,000). IR (KBr): 3455 cm^{-1} (ν OH); 1604 cm^{-1} (C=C); 1258 cm^{-1} (ν CO). ^{1}H NMR ($CDCl_3$) δ: −1.65 (s, 2H, NH); 4.16 (s, 4H) 7.09 (m, 8H); 7.52 (m, 8H); 8.24 (d, 2H); 8.36 (s, 2H); 8.63 (d, 2H); 9.73 (s, 2H, OH); 9.79 (s, 2H, OH). MS-ESI$^+$ (flow injection): *m/z* 681.6 (100%); 682.5 (45%); MS-ESI$^-$ (flow injection): *m/z* 679.4 (100%). Molecular weight calculated for $C_{44}H_{32}N_4O_4$ = 680.8.

6.2. 5,10,15,20-Tetra(4-hydroxyphenyl)-2,3-dihydro-21H,23H-chlorin [H_2-T(*p*-OH)P-CL, 9]

The same procedure described for compound **8** was used for this compound, thus 160 mg (0.236 mmol) of porphyrins **6** in 10 mL of pyridine was treated with 131.8 mg (0.708 mmol) of toluene-4-sulfonylhydrazide and 163 mg (1.18 mmol) of K_2CO_3. The reaction was followed as described above and, at the end, the title compound was isolated as a solid product (109 mg, 67.7%). UV–vis$_{(water)}$: 424 nm (ε = 64,000); 656 nm (ε = 10,100). IR (KBr): 3409 cm^{-1} (ν OH); 1601 cm^{-1} (ν C=C); 1255 cm^{-1} (ν CO). ^{1}H NMR ($CDCl_3$) δ: −1.56 (s, 2H, NH); 4.11 (s, 4H); 7.12 (dd, 8H); 7.67 (d, 4H); 7.86 (d, 4H); 8.20 (d, 2H); 8.35 (s, 2H); 8.60 (d, 2H); 9.74 (s, 2H, OH); 9.87 (s, 2H, OH). HPLC: retention time = 4.54. MS-ESI$^+$: *m/z* 681.4 (100%); 682.4 (40%); MS-ESI$^-$ *m/z* 679.3 (100%). Molecular weight calculated for $C_{44}H_{32}N_4O_4$ = 680.8.

6.3. 5,10,15,20-Tetra(3,4,5-trimethoxyphenyl)-2,3-dihydro-21H,23H-chlorin [H_2-T(OMe)$_3$P-CL, 10]

A solution of 40 mg (0.041 mmol) of porphyrins **3** in 5 mL of pyridine was treated with 30.5 mg (0.164 mmol) of toluene-4-sulfonylhydrazide and 56.6 mg (0.41 mmol) of K_2CO_3. The mixture was refluxed for 2 h. After this period the reaction was checked through UV–vis every hour and the same amount of hydrazide and K_2CO_3 was added until the reaction was completed. Then 15 mL of AcOEt and 8 mL of H_2O were added and the mixture was kept at 70 °C for 1 h. The organic phase was isolated, washed (HCl 10%, then H_2O), dried (Na_2SO_4), and concentrated affording 38 mg (92%) of a solid product.

UV–vis$_{(water)}$: 424 nm (ε = 93,000); 630 nm (ε = 14,500). IR (KBr): 2931 cm^{-1} (ν CH); 1127 cm^{-1} (CO). ^{1}H NMR ($CDCl_3$): −1.47 (s, 2H, NH); 3.94 (s, 12H, OCH_3); 3.96 (s, 12H, OCH_3); 4.14 (s, 6H, OCH_3); 4.16 (s, 6H, OCH_3); 4.28 (s, 4H); 7.10 (s, 4H); 7.39 (s, 4H); 8.11 (d, 4H). HPLC: retention time = 2.62 min. MS-APCI$^+$: *m/z* 977.6 (100%); 978.6 (40%); 979.5 (10%); MS-APCI$^-$: *m/z* 976.5 (100%); 977.4 (55%);. Molecular weight calculated for $C_{56}H_{56}N_4O_{12}$ = 977.1. Anal. Calcd: C, 68.83; H, 5.77; N, 5.73. Found; C, 69.38; H, 5.75; N, 5.62.

6.4. 5,10,15,20-Tetra(3,4,5-trihydroxyphenyl)-2,3-dihydro-21H,23H-chlorin [H_2-T(OH)$_3$P-CL, 11]

A solution of 20 mg (20 mmol) of chlorin **10** in 8 mL of CH_2Cl_2 was treated with 0.6 mL (0.6 mmol) of BBr_3 solution as described above. The insoluble product was recovered by filtration and purified by gel permeation chromatography on Bio-Beads resins, with CH_3CN as eluant, yielding 10 mg (62.5%) of the desired product.

UV–vis$_{(water)}$: 420 nm (ε = 77,000); 638 nm (ε = 12,000). IR (KBr): 3490 cm^{-1} (ν OH); ^{1}H NMR (DMSO): −1.50 (s, 2H, NH); 4.06 (s, 4H); 7.50 (m, 4H); 7.80 (m, 4H); 8.40 (s, 6H) 9.60 (m, 12H, OH). MS-ESI$^+$: *m/z* 809.5 highest peak of molecular cluster. Molecular weight calculated for $C_{44}H_{32}N_4O_{12}$ = 803.7. Anal. Calcd: C, 65.76; H, 4.01; N, 2.24. Found; C, 66.23; H, 4.01; N, 2.28.

6.5. 5,10,15,20-Tetra(4-*N*-ethoxyethanolsulfonamidophenyl)-2,3-dihydro-21H,23H-chlorin [H_2-T(*p*-SO_2 AEE)P-P, 12]

The same procedure described for compound **10** was used for this compound, thus 80 mg (62 mmol) of porphyrins **4** in 7 mL of pyridine was treated with 34.6 mg (186 mmol) of toluene-4-sulfonylhydrazide and 43 mg (310 mmol) of K_2CO_3. The reaction was followed as described above, and the title compound was isolated as a solid product (42 mg, 52%).

UV–vis$_{(water)}$: 420 nm (ε = 72,000); 650 nm (ε = 11,000). IR (KBr): 3430 cm^{-1} (ν OH); 1610 cm^{-1} (ν C=C). ^{1}H NMR (DMSO) δ: −1.50 (s, 2H, NH); 3.15 (t, 8H), 3.40 (m, 8H), 3.45 (m, 20H), 4.20 (s, 4H); 4.66 (br s, 4H), 7.55 (d, 8H), 7.95 (d, 8H); 8.25 (d, 2H), 8.34 (d, 2H), 8.60 (d, 2H). MS-MALDI-TOF: *m/z* 1284.9 highest peak of molecular cluster. Molecular weight calculated for $C_{60}H_{68}N_8O_{16}S_4$ = 1285.5. Anal. Calcd: C, 56.05; H, 5.33; N, 8.71. Found; C, 55.71; H, 5.40; N, 8.62.

6.6. Cytotoxicity studies

Human adenocarcinoma HCT116 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in DMEM (Mascia-Brunelli, Milano, Italy) supplemented with 10% fetal bovine serum (Mascia Brunelli) at 37 °C in a humidified 5% CO_2 atmosphere. The antiproliferative effects of the different PSs, including Photofrin, were assessed using the MTT assay.[19] 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. After 24 h, the PS-containing medium was replaced by PBS, and cells were irradiated under visible light (halogen lamp 500 W) for 2 h (average value between 400 and 700 nm determine with a LICOR-1800 spectroradiometer; light irradiance 5.5×10^{-2} mW cm^{-2} nm^{-1} and a light energy of 39.6 mJ cm^{-2} nm^{-1}). At the end of this time cells were incubated for 24 h 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). Possible intrinsic (i.e., nonphotodynamic) cytotoxic effects of the PS were assessed on control treated as described above, but omitting cell irradiation.

IC_{50} (dose affecting 50% of cells) values were obtained by nonlinear regression analysis, using the GraphPad PRISM 3.03 software (GraphPad Software Inc., San Diego CA).

7. Visualization of apoptotic cells

Apoptotic cells were visualized by fluorescence microscopy. HCT116 cells were seeded onto coverslips, allowed to grow for 48 h and subsequently treated with the different PSs, at concentrations approximating their respective IC_{50} values. After 24 h, the PS-containing medium was replaced by PBS, and cells were irradiated for 2 h, as previously described. At the end of this time cells were incubated for 24 h in drug-free medium. Cells still attached to coverslips were washed in phosphate-buffered saline (PBS) and fixed in 70% methanol for at least 30 min at −20 °C, washed in PBS, and incubated in PBS containing RNAse A (1 kU/mL), propidium iodide (50 mg/mL), and 0.05% Nonidet (N)P40. After 30 min on ice, coverslips were mounted onto glass slides with Mowiol 4-88 and observed with an Olympus photomicroscope equipped for epifluorescence.

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