THE EFFECT OF SUBSTITUENTS ON PHTHALOCYANINE PHOTOCYTOTOXICITY

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Abstract—Phthalocyanine (Pc) dyes are a class of photosensitizers that are being considered for use in the photodynamic therapy of cancer. A final choice for the most useful drug demands phototoxicity screening of various structurally related Pc dyes, such as those with different substituents at the peripheral benzene rings. Using the colony forming ability of Chinese hamster cells as an endpoint, the photobiological activity for ZnPc was found to be the highest for the unsubstituted compound, followed in decreasing order by the 2,9,16,23-tetrahydroxy-, 2,9,16,23-tetrasulfonated-, and 2,9,16,23-tetraneopentoxy-Pc derivatives. This effect could be correlated with the uptake rate of the respective Pc derivatives by the cells, but it is unrelated to the apparent lipophilicity of these compounds, or, alternatively, to their ability to photogenerate singlet oxygen. The first synthesis of a metal-free tetrahydroxy Pc is described.

INTRODUCTION

The potential use of Pc† dyes for photodynamic therapy of cancer has sparked a considerable interest in photobiology of these compounds, from reactions with biochemicals (Spikes and Bommer, 1986; Langlois *et al.*, 1986; Wagner *et al.*, 1987) and enzyme inactivation (Robinson *et al.*, 1987), to destruction of cultured cells (Ben-Hur and Rosenthal, 1986; Brasseur *et al.*, 1985; Chan *et al.*, 1986), sensitization of organs in animals (Bown *et al.*, 1986), and eradiction of tumors in animals (Selman *et al.*, 1986; Brasseur *et al.*, 1987). A review of the earlier work on phthalocyanines as photosensitizers has appeared (Spikes, 1986).

In view of this recent history for use of Pc dyes as photobiological sensitizers, it is still premature to define the structural requirements for the ideal photochemotherapeutical agent of this class. As a matter of fact only one criterion has yet emerged for selecting an efficient sensitizer. That is the requirement for a reasonable long life-time excited triplet state so that its reactions are facilitated. Thus Pc containing paramagnetic metals such as copper or cobalt, which for manufacturing convenience have been the most common Pc used as pigments, are not suitable for this application (Rosenthal *et al.*, 1986).

It was the aim of this study to screen the photoefficiency of various ZnPc substituted in the benzene rings (Fig. 1) using the colony forming ability of Chinese hamster cells as an endpoint. This chemical modification can drastically affect the solubility, and consequently the hydrophilicity–lipophilicity of the sensitizer which might markedly affect the photobiological activity. We note that optimization of the porphyrin anti-tumoral activity has been suggested to require hydrophobic sensitizers characterized by high yields in $^1O_2$ photoproduction (Emiliani and Delmelle, 1983).

Although ZnPc (**1**) is commercially available, and tetrasulfonated derivative (**2**) has been known, the synthesis of a highly lipophilic ZnPc such as 2,9,16,23-tetraneopentoxyPc (**3a**) and its zinc (II) derivative (**3b**) has only recently been reported (Leznoff *et al.*, 1984, 1985). In addition, 2,9,16,23-tetrahydroxyPc (**4a**) has not been prepared, although some of its metallated derivatives have been mentioned but not characterized (Borisenkova *et al.*, 1975). It was believed that a 2,9,16,23-tetrahydroxyZnPc (**4b**) would be particularly interesting to test due to its potential solubility and hydrogen-bonding characteristics.

MATERIALS AND METHODS

Sensitizers. ZnPc (**1**) was purchased from Eastman Kodak Co. (Rochester, NY), and used as supplied. ZnPc tetrasulfonate Na salt (**2**) was synthesized by condensation of 4-sulfophthalic acid (Aldrich Chemical Co., Milwaukee, WI) in the presence of $ZnSO_4$ (Weber and Busch, 1965). 2,9,16,23-TetraneopentoxyPc (**3a**) and its zinc derivative (**3b**) were prepared as previously described (Leznoff *et al.*, 1985).

Preparation of 2,9,16,23-tetrahydroxyPc (**4a**). To 3.02 g (3.52 mmol) 2,9,16,23-tetraneopentoxyPc (**3a**) in 100 mℓ of dry benzene was added 2.3 mℓ (24.19 mmol) of boron tribromide in two portions. After 20 h reflux an additional 0.5 mℓ of boron tribromide was added and the reflux was continued for 4 h. After cooling at room temperature, 30 mℓ water was added and the solvent was evaporated under

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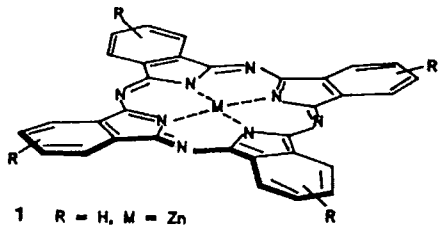
†*Abbreviations*: Pc-phthalocyanine; PBS-phosphate buffered saline; DMEM-Dulbecco's modified Eagle's medium.



1 R = H, M = Zn

2 R = SO_3H, M = Zn

3a R = $OCH_2C(CH_3)_3$, M = H_2

3b R = $OCH_2C(CH_3)_3$, M = Zn

4a R = OH, M = H_2

4b R = OH, M = Zn

Figure 1. The molecular structure of metallophthalocyanine.

reduced pressure. The crude product was filtered and washed with water until the filtrate was colorless. The residue was then dried and continuously extracted with toluene in a Soxhlet apparatus for 15 h until the extract was clear. This process removed some uncleaved and partially cleaved products. After drying overnight at 50°C, 1.62 g was recovered (80%). A sample of 50 mg was further purified by flash chromatography (Still *et al.*, 1978) using 1 cm wide column packed with silica gel 4 cm high. The product was preadsorbed on silica and eluted under Ar with dry, freshly distilled 2-methoxyethanol followed by increasing amounts of acetic acid in 2-methoxyethanol (1 : 9 to 1 : 1) to yield 26.4 mg of **4a** as dark blue shining solid (53% recovery). On a larger scale (1.26 g) the recovery from the column was 22%. Absorption spectrum: λ_{max} (2-methoxyethanol) 708, 672, 644, 614, 390, 342, 292, 232, 218 (log ϵ: 4.99, 4.93, 4.61, 4.42, 4.51, 4.81, 4.63, 4.55, 4.55). ^{1}H NMR spectrum (300 MHz, DMSO d_6): δ−3.1 (broad signal) NH, 7.43, 8.10 and 8.48 (broad signals) 12 H, 10.45 (broad signal) OH. IR spectrum (KBr pellet): broad band from 3100 to 3500 cm^{-1}, 1610, 1480, 1310, 1200 to 1250, 1095, 1010, 930, 820, 740 and 710 cm^{-1}. Mass spectrum: (M^+), *m/z* = 578. Analysis: $C_{32}H_{18}N_8O_4$. Calc. (%): C (66.43), H (3.14). N (19.37). Found (%): C (66.20), H (3.40), N (19.20).

Preparation of 2,9,16,23-tetrahydroxyZnPc (**4b**). To 1.58 g (1.72 mmol) of 2,9,16,23-tetraneopentoxyZnPc (**3b**) in 50 mℓ of dry benzene, was added 1.5 mℓ (15.90 mmol) of boron tribromide. After 20 h reflux under Ar the mixture was cooled, 20 mℓ water was added and the solvent was evaporated under reduced pressure. The crude product was filtered and washed with water until the filtrate was colorless. The residue was then extracted with toluene in a Soxhlet apparatus. The extract was completely clear indicating that cleavage is complete. Further purification of the product was achieved by flash chromatography (Still *et al.*, 1978) using 2 cm wide column packed with silica gel 6 cm high. The product was preadsorbed on silica and eluted with dry methanol under Ar to give 1.03 g (94%) of 4b as dark blue shining solid. Absorption spectrum: λ_{max} (ethanol) 684, 672, 616, 348, 288, 234, 212 nm (log ϵ: 5.00, 4.90, 4.44, 4.83, 4.52, 4.59, 4.61). ^{1}H NMR spectrum (300 MHz, DMSO d_6): δ−7.63 (broad signal) 1H, 8,67 (broad signal) 1H, 9.12 (broad signal) 1H, 10.60 (broad signal) OH. IR spectrum (KBr pellet): broad band from 3100 to 3500 cm^{-1}, 1600, 1475, 1300, 1240, 1085, 1040, 945 and 745 cm^{-1}. Mass spectrum: *m/z* 640, 641, 642, 643, 644, 645. Analysis: $C_{32}H_{16}N_8O_4Zn$. Calc. (%): C (59.88), H (2.51), N (17.46), Zn (10.18). Found (%): C (59.64), H (2.90), N (16.79), Zn (9.64).

Alternatively **4b** was obtained from **4a** as follows: To 25.2 mg (0.044 mmol) of **4a** in 1.2 mℓ of dry dimethylformamide (DMF) was added 20.0 mg (0.14 mmol) of zinc acetate and the mixture was heated under Ar to 150°C (oil bath) for 20 h. After cooling to room temperature the DMF was removed under reduced pressure (1 mm Hg) and the crude product was dissolved in dry methanol and flushed under Ar through a short silica gel column (1 × 4 cm). Elution with dry methanol yielded 22.8 mg (82%) of **4b**.

Chinese hamster cells. Chinese hamster fibroblasts, line V79-B310H were grown as a monolayer in glass or plastic Petri dishes using DMEM containing 10% fetal calf serum. The cells doubled in number every 9 h at 37°C in a humidified atmosphere containing 5% CO_2. Survival of the log-phase cells was determined using colony-forming ability as an endpoint (Ben-Hur *et al.*, 1978). Briefly, cells were plated in 50 mm plastic dishes at a concentration of 200–20 000 per dish and were grown overnight with or without added dye prior to light exposure. As a result, cellular multiplicity at the time of tratment was *ca.* 3. Each datum point is the average of three dishes. Standard errors are shown where larger than the symbols. The results shown are of individual experiments. Each experiment was repeated twice. Differences between experiments were not statistically significant ($P < 0.05$).

Dye uptake. The various Pc derivatives were added into the culture medium of log-phase Chinese hamster cells in 100 mm Petri dishes containing 5 × 10^6 cells/dish. After various incubation times at 37°C the cells were rinsed three times with cold PBS, air dried and then extracted 1 h at room temperature with 4 mℓ extracting solution. The extracting solvents were dimethylformamide for ZnPc, ethanol for tetrahydroxyZnPc, acetone for neopentoxyZnPc and 0.1 N NaOH containing 1% cetrimide for tetrasulfonatedZnPc. The absorption spectra of the cell extracts were recorded with a Gilford 2600 spectrophotometer. The amount of Pc in the cell extract was calculated from the absorption at λ_{max} using standard curves.

Light exposure. Prior to light exposure the growth medium was removed and 3 mℓ PBS were added to each dish. The dishes were then exposed at room temperature to white light from a bank of three 40 W "cool light" fluorescent tubular lamps (Sylvania) held in a reflector. The total light fluence rate was 55 W m^{-2} at the level of the cell monolayer, of which *ca.* 15% was in the wavelength range of 600–700nm.

EPR measurements and determination of quantum yields for singlet oxygen generation. A Varian E-9, X-band spectrometer was used. The magnetic field modulation frequency was 100 kHz. The scans were traced with a modulation amplitude equal to or lower than 0.2 G, and the microwave power level was maintained at 10 mW to avoid saturation. The irradiations were performed *in situ* at room temperature in a standard quartz cell (60 × 10 × 0.25 mm) placed in the EPR cavity, using a Schoeffel 1000 W high-pressure Hg–Xe lamp coupled with a Schoeffel grating monochromator (estimated bandwidth 20 nm) and a timer-activated shutter. All the Pc tested were irradiated with monochromatic visible light at the lowest energy absorption band, in an oxygen-saturated solution of 2,2,6,6-tetramethyl- 4-piperidone (0.3 *M*), and the paramagnetic signal was recorded *vs.* irradiation time. In an oxygen-free solution the formation of the nitroxide radical was inhibited. The dye concentration was high enough to ensure total absorption of the light. The oxidation of the amine was linear in time for the duration of the experiment (10 min) and the relative reaction rates could be estimated from the slopes of the plots and reflect the relative efficiency for $^1O_2$ generation. The absolute quantum yields for $^1O_2$ production were calculated from these rates relative to that for a sensitizer of known quantum yield, Rose Bengal, which was subjected to the same technique for

detection of $^1O_2$. The Rose Bengal quantum yield was taken as 0.75 (Gandin *et al.*, 1983) for λ_{exc} = 550 nm. The relative number of incident photons at 550 nm and λ_{exc} of each dye was determined using a lightmeter and these values were used in the calculation of the quantum yield.

RESULTS AND DISCUSSION

2,9,16,23-TetrahydroxyPc (**4a**) had not been reported prior to this study possibly due to the fact that the normal precursor to **4a**, 4-hydroxyphthalonitrile does not undergo condensation to **4a**. The desired **4a** could be prepared by ether cleavage of tetraalkoxy Pc's such as **3a** and **3b**. Thus cleavage of **3a** with boron tribromide (BBR_3) (McOmie and Watts, 1963) in refluxing benzene for 20 h liberated **4a** in 80% yield as a mixture of isomers of which 2,9,16,23-tetrahydroxyPc (**4a**) is the typical substitution pattern. The other isomers of 2–4 would only include the very similar 2,9,16,24- 2,9,17,24-, and 2,10,16,24-derivatives. The tetrahydroxyPc (**4a**) could be converted to the 2,9,16,23-tetrahydroxy zinc (II) derivative (**4b**) by treating **4a** with $Zn(OAc)_2$ in dimethylformamide (DMF) at 150°C. Preferably, **4b** could be obtained by direct cleavage of the 2,9,16,23-tetraneopentoxyZnPc (**3b**), in 90% yield. The metal is retained under the vigorous conditions of ether cleavage and **4b** is more easily purified than **4a** by chromatographic methods.

While phototoxicity of the traditional photosensitizer for photodynamic therapy, that is hematoporphyrin derivative, has been mechanistically explained by a singlet oxygen reaction (Dougherty *et al.*, 1982), we could not find yet any confirming evidence that this is true for Pc dyes as well. However, it seems that only a Pc dye which can populate the triplet excited state for sufficiently long time, which is by itself a preliminary requirement for generation of singlet oxygen, can act as a photodynamic sensitizer (Rosenthal *et al.*, 1986). We therefore estimated the quantum yield for singlet oxygen generation by three out of four Pc derivatives tested in this study: tetrasulfonatedZnPc, tetrahydroxyZnPc and tetraneopentoxyZnPc. The low solubility of the unsubstituted ZnPc prevented a quantitatively meaningful determination for this compound. The singlet oxygen production was estimated by electron paramagnetic resonance, monitoring the conversion of a sterically hindered heterocyclic amine, 2,2,6,6-tetramethyl-4-piperidone to the corresponding N-oxide radical. This stable radical can be easily detected at ambient temperature as a 1 : 1 : 1 triplet of a_N = 16.5 G (Cannistraro *et al.*, 1978; Moan and Wold 1979). Due to the different solubilities, the measurements had to be performed in different solvents: water for tetrasulfonatedZnPc, ethanol and acetone for tetrahydroxyZnPc, and acetone for tetraneopentoxyZnPc. All three dyes photosensitized the oxidation of 2,2,6,6-tetramethyl-4-piperidone to the N-oxide radical with the same reaction rate. Since the solvent-induced radiationless decay constants of singlet oxygen are different for the solvents employed [k_d (water) = 5×10^5 s^{-1}; k_d (ethanol) = 4×10^5 s^{-1}; k_d (acetone) = 3.8×10^4 s^{-1}] (Wilkinson and Brummer, 1982), the oxidation rate would be affected if the solvent-induced decay limits the singlet oxygen lifetime in the system. Such an effect could not be observed in the comparative tests with tetrahydroxyPc in ethanol and acetone under our experimental conditions. Since the oxidation rates were the same, it means that tetrahydroxyZnPc and tetraneopentoxyZnPc generate $^1O_2$ with the same quantum yield. Assuming also the absence of a solvent effect for the tetrasulphonatedZnPc in water, the quantum yields for $^1O_2$ generation by all three Pc tested could be estimated to be the same: 0.45 ± 0.05. Since, however, the rate constants for photooxidation of an analogous amine, 2,2,6,6-tetramethyl-4-piperidinol with singlet oxygen, in ethanol and aqueous buffer were reported 8×10^5 $M^{-1}s^{-1}$ and 4×10^7 $M^{-1}s^{-1}$, respectively, (Lion *et al.*, 1980), the quantum yields of tetrahydroxy- and tetraneopentoxyZnPc could be greater.

These measurements indicate that all three dyes in the excited triplet state can interact with a ground state molecule and can generate singlet oxygen. *A priori*, these results suggest that all these compounds could be efficient biological photosensitizers. [We consider this rapid test as a useful preliminary screening for photodynamic activity of a new sensitizer; for example the biological inactive phthalocyanines containing paramagnetic metals (Ben-Hur and Rosenthal, 1986) could not photooxidize 2,2,6,6-tetramethyl-4-piperidone].

The effect of various substituents on the benzene rings of ZnPc on the photoinactivation of Chinese hamster cells is shown in Fig. 2. Thus the unsubstituted ZnPc is the most effective sensitizer, followed by the tetrahydroxy and tetrasulfonate derivative. The neopentoxy substitution essentially abolishes the phototoxicity in spite of the ability of this dye

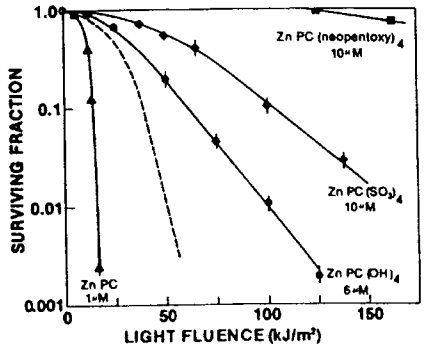


Figure 2. Survival of Chinese hamster cells photosensitized by Pc derivatives. Cells were incubated in growth medium with the indicated dyes for 16 h and then exposed to light. The dashed curve is for 10 μM ClA1Pc$(SO_3Na)_4$.

to generate singlet oxygen. It is noted that the absence of a direct correlation between cell killing and ability to photooxidize a model compound has also been reported for several sulphonated Pc having different central atoms (Langlois *et al.*, 1986).

It is noted that the phototoxicity of tetrahydroxyZnPc was unchanged over the pH range 6 to 8.

The rate at which photosensitivity develops is illustrated in Fig. 3. The kinetics are relatively rapid for ZnPc and tetrahydroxyZnPc during the first 2 h of incubation. At longer times, further sensitivity develops very slowly. Photosensitivity to tetrasulfonatedZnPc appears slowly over many hours and begins to plateau only after 24 h.

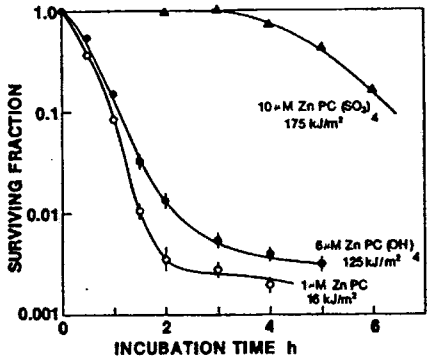


Figure 3. Kinetics of photosensitization of Chinese hamster cells by ZnPc derivatives. The cells were incubated with the indicated dyes for various periods of time in DMEM containing 10% serum and then exposed to the indicated light fluences.

The kinetics of photosensitivity appearance are correlated with the rate of uptake of the various derivatives (Fig. 4). Thus ZnPc is taken up at the fastest rate, followed by tetrahydroxy-, the tetrasulfonated- and tetraneopentoxy derivatives, respectively. The rate for ZnPc and tetrahydroxyZnPc is fast during the first 2 h, and becomes slow at later times. The rate of uptake of the tetrasulfonate is linear up to 24 h.

All these four Pc dyes were tested for binding to bovine serum albumin (Ben-Hur and Rosenthal, 1986). Unlike the other three derivatives, tetraneopentoxyZnPc does not bind to protein at all and this might explain the absence of a significant cellular uptake. The absence of binding to protein could be due to the steric hindrance induced by the bulky neopentoxy groups.

The uptake rate cannot be correlated to any solubility trends of these sensitizers. Thus the least active tetraneopentoxyZnPc is the most lipophilic Pc compound known; highly soluble in hexane and acetone (at least 10^{-2} *M*) it is insoluble in water. On the other hand, tetrahydroxyZnPc while soluble

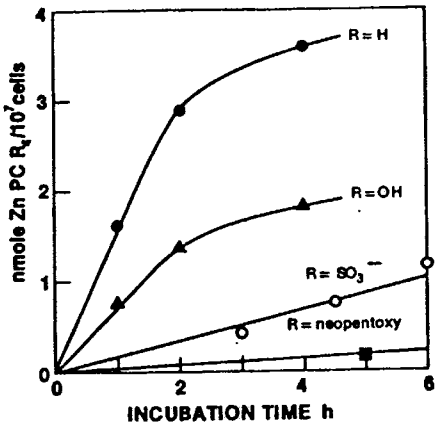


Figure 4. Uptake of ZnPc derivatives into Chinese hamster cells. The dyes were added to log-phase cells at 10 μ*M* ZnPc(●), 25 μ*M* ZnPc(OH)$_4$ (▲), 25 μ*M* ZnPc(SO$_3$Na)$_4$ (○), and 25 μ*M* ZnPc (Neopentoxy)$_4$ (■). After various times at 37°C the amount of dye taken up was measured as described in Materials and Methods.

in ethanol and acetone (at least 10^{-2} *M*) is only hardly soluble in water (less than 10^{-6} *M*). The tetrasulfonated derivative is the most water-soluble (at least 10^{-2} *M*) and slightly soluble in ethanol (less than 10^{-4} *M*). Finally, the unsubstituted ZnPc, while insoluble in water, ethanol or acetone is soluble only in highly polar solvents such as dimethylformamide or dimethylsulfoxide.

Thus, while cellular uptake, as expected, is a determining factor in the subsequent phototoxicity, it cannot be predicted from the solubility features of the Pc dyes.

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