SULFONATED PHTHALIMIDOMETHYL ALUMINUM PHTHALOCYANINE: THE EFFECT OF HYDROPHOBIC SUBSTITUENTS ON THE *in vitro* PHOTOTOXICITY OF PHTHALOCYANINES

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Abstract—The photocytotoxicity of sulfonated phthalimidomethyl aluminum phthalocyanine, a more hydrophobic photosensitizer as compared to phthalocyanine substituted with sulfonate groups only, was investigated. Inclusion of 1–2 phthalimidomethyl groups into disulfonated aluminum phthalocyanine, resulted in increased partition coefficients between *n*-octanol and water, and a six-fold increase in both cellular uptake and photocytotoxicity towards Chinese hamster lung fibroblast cells (line V-79). Reducing the number of phthalimidomethyl groups, or increasing the degree of sulfonation, lead to a decrease in the partition coefficient, cellular uptake, and phototoxicity. The quantum yield of singlet oxygen was comparable for all dyes tested in this series, indicating that no significant change in this photophysical parameter resulted from phthalimidomethylation. These results suggest that the addition of 1–2 phthalimidomethyl groups to disulfonated aluminum phthalocyanine improves cellular uptake, but, as the relative efficiency of cell killing was not effected, the intracellular distribution on photosensitive molecules may not be modified.

INTRODUCTION

Photodynamic therapy (PDT)† has been proposed as an alternative therapy of neoplastic disease (Dougherty *et al.*, 1978) and due to the advantageous relationship between the absorption maximum and light penetration in tissue, the phthalocyanines are studied as potential photosensitizers for use in this treatment (for recent reviews see Rosenthal and Ben-Hur, 1989; van Lier and Spikes, 1989; van Lier, 1990). These well-defined compounds, when sulfonated to render them water soluble, and chelated with an appropriate metal in order to maximize generation of the reactive species singlet oxygen (Girotti, 1983; Langlois *et al.*, 1986) efficiently destroyed transformed cells (Brasseur *et al.*, 1985, 1987; Ben-Hur and Rosenthal, 1985; Chan *et al.*, 1986). Using V-79 Chinese hamster cells, amphiphilic phthalocyanines with two sulfonate groups on adjacent benzenoid rings interacted with the lipid portion of the plasma membrane and were shown to enter into the cytoplasm after 24 h incubation *in vitro* (Paquette *et al.*, 1988). This amphiphilic property explained their high photocytotoxicity under *in vitro* conditions.

In vivo, using Photofrin II or phthalocyanines,

necrosis mainly results from tumor microcirculation stasis (Fingar and Henderson, 1987; Selman *et al.*, 1985; Reed *et al.*, 1989). Such an indirect effect on the tumor could allow tumor regrowth unless normal blood vessels surrounding the tumor were also destroyed (Star *et al.*, 1986). The effect also appeared to be non-specific as both normal and tumoral vessels were damaged to a similar degree (Reed *et al.*, 1989). Among the sulfonated aluminum phthalocyanines (AlOH-PcS) the lower sulfonated amphiphilic dyes have a superior potential for direct tumor cell inactivation while being relatively sparing to the vasculature (Henderson and Bellnier, 1989).

With the aim of augmenting direct tumor response on neoplastic cells, we synthesized a more hydrophobic sulfonated aluminum phthalocyanine by introducing phthalimidomethyl groups concurrently at the sulfonation stage (Fig. 1). In this study we report the partition coefficient, cellular uptake, photocytotoxicity, and quantum yield of single oxygen for sulfonated phthalimidomethyl aluminum phthalocyanine (AlOH-PcSP), and compare these results with those obtained for unmodified AlOH-PcS.

MATERIALS AND METHODS

Synthesis. Sulfonated aluminum phthalocyanine was prepared as previously described (Ali *et al.*, 1988). Sulfonated phthalimidomethyl aluminum phthalocyanine was prepared as follows: 1.34 g (2.32 mmol) of aluminum phthalocyanine (Ciba Geigy) was added to 5 mL of concentrated sulfuric acid, the mixture was stirred for 20 min

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† *Abbreviations*: AlOH-PcS, sulfonated aluminum phthalocyanine; AlOH-PcSP, sulfonated phthalimidomethyl aluminum phthalocyanine; LDL, low density lipoprotein; PBS, phosphate-buffered saline; PDT, photodynamic therapy; ϕ_Δ, quantum yield of singlet oxygen.
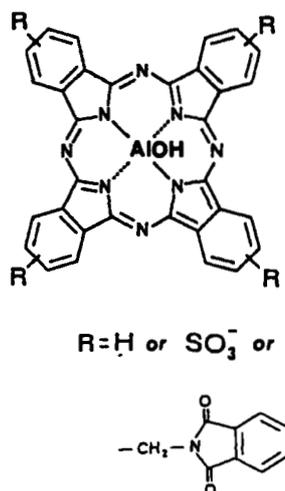
Figure 1. Chemical structure of sulfonated phthalimido-methyl aluminum phthalocyanines (AlOH-PcSP). $R = H$, SO_3^- or CH_2-phthalimide, depending on the degree of substitution and sulfonation.

then 0.41 g (2.79 mmol) phthalimide, 0.12 g (4 mmol) *par-aformaldehyde*, and 15 mL of oleum containing 30% free SO_3 were added. The mixture was stirred at 80°C for 5 h. The reaction mixture was poured on crushed ice (200 mL) and the resulting precipitate recovered by filtration, washed with 1 M H_2SO_4 (200 mL), redissolved in 1 M KOH and filtered again before being neutralized. After removal of water by evaporation *in vacuo*, salt was removed from the dried product by dissolution in dry methanol and filtration to yield AlOH-PcSP (1.67 g). Molar absorption coefficients of the AlOH-PcSP (ϵ_{MeOH} = 1.5×10^5 at λ_{max} = 676 nm) were slightly lower than those found for the AlOH-PcS ($\epsilon_{MeOH} = 1.9 \times 10^5$ at λ_{max} = 674 nm).

Fractionation of sulfonated phthalimidomethyl aluminum phthalocyanine (AlOH-PcSP). "Crude" AlOH-PcSP (1.1 g) was fractionated by preparative medium pressure reverse phase chromatography on a 30 cm long by 2 cm ID glass column packed with C-18 reverse phase, particle size 25–40 μm (Macherey-Nagel, Düren, Germany) using a linear gradient from 0 to 95% MeOH in 10 mM sodium phosphate buffer, pH 5 (Ali *et al.*, 1988). Samples were analyzed by reverse phase HPLC, eluting phthalocyanines being detected spectroscopically at 670 nm, and pooled to yield six main fractions (A–F). The relative level of substitution was determined by oxidative degradation of 1 mg of compound using cerium ammonium nitrate (0.01 mmol; 4–5 mL) in hot, acidic aqueous solution, followed by reverse phase HPLC analysis (Ali *et al.*, 1988). The eluting phthalimide, and substituted phthalimide units, resulting from the oxidation, were detected spectroscopically at 215 nm. The identity of phthalimide degradation products was determined by coupled HPLC/mass spectrometry. Unsulfonated material (phthalimide R_t = 29 min; phthalimidomethylphthalimide R_t = 35 min) was characterized by direct comparison of chromatographic properties with those of authentic samples, whereas the sulfonated moieties were found indirectly by HPLC comparison of AlCl-PcP and AlOH-PcSP degradation fragments (sulfophthalimide R_t= 16 min; sulfophthal-imidomethylphthalimide R_t = 20 min). Under the reaction conditions some sulfonation of the benzenoid ring of the phthalimidomethyl group also resulted. The level of this sulfophthalimidomethyl moiety in each fraction was found to be constant with a relative value of 0.2 mol per mol Pc which was taken into account as sulfonate substitution in the molar composition of the various frac-

tions. In this manner the following compositions were assigned to fractions A–F: A (R_t = 18 min), AlOH-PcS$_{3.7}$P$_{0.1}$; B (R_t = 20 min), AlOH-PcS$_{3.2}$P$_{0.2}$; C (R_t = 25 min), AlOH-PcS$_{3.0}$P$_{0.3}$; D (R_t = 28–33 min), AlOH-PcS$_{3.2}$P$_{0.4}$; E (R_t = 37 min), AlOH-PcS$_{2.6}$P$_{0.6}$; F (R_t = 40 min), AlOH-PcS$_{2.4}$P$_{1.2}$. For the *in vitro* studies only fractions D and F were evaluated.

Partitioning studies. Dyes (25–300 nM) were partitioned between *n*-octanol and 100 mM Tris buffer pH 7.4. After shaking for 2 min, the phases were separated by centrifugation. The dye concentration in each phase was measured by fluorescence, after 40-fold dilution in 100% methanol, using a SLM-Aminco SPF-500C spectrofluorometer (AlOH-PcS: λ_{ex} = 664 nm, λ_{em} = 684 nm; AlOH-PcSP: λ_{ex} = 666 nm, λ_{em} = 686 nm).

Cell uptake. 3×10^6 Chinese hamster lung fibroblasts cells (line V-79) were plated in 60 mm Petri dishes. After an incubation period of 3 h under 5% CO_2, to allow cell attachment, 10 μM dye in medium (1 mL) containing 1% serum was added and the cells were incubated for 1 h. Absorbed dyes were extracted and quantified as previously described (Paquette *et al.*, 1990). Briefly, after removal of the medium, cells were washed three times with PBS and detached with 600 μL trypsin-EDTA 0.25% (4–5 min incubation) and dishes were washed with 600 μL PBS. Cells were collected by centrifugation in 1.5 mL plastic tubes (5 min, 600 g) and resuspended in 200 μL of buffer (Tris 0.2 M, MgCl$_2$ 10 mM, CaCl$_2$ 1 mM, pH 7.8). After three freeze–thaw cycles in liquid nitrogen, cell debris was incubated in the dark, overnight, with 0.5 A$_{280}$ unit of proteinase K (Sigma Chemical Co, St. Louis, MO) and diluted in 100 μL of Tris buffer. DNA was digested for 2 h at 37°C with 1 A$_{280}$ unit of micrococcal nuclease (Worthington, Freehold, NJ). To eliminate hydrophobic and ionic interactions with cellular fragments, 50 μL DMF was added, whereafter the mixture was incubated for 30 min followed by the addition of 50 μL of 1.0 N NaOH and a 10 min incubation period at 37°C. After 40-fold dilution in 100% methanol, dye concentrations were measured by fluorescence as described above.

Photocytotoxicity assay. Cell survival of Chinese hamster lung fibroblasts (line V-79) was determined using a colony forming assay as described by Brasseur *et al.* (1985). After the attachment of 200 cells in Petri dishes, the cells were rinsed with PBS, and incubated for 1 h with 1 mL of medium containing 1% serum and the dye. After removal of the dye and washing with PBS, the cells were exposed at room temperature for 4 min to red light. The irradiation device consisted of a 500 W tungsten/halogen lamp equipped with a 10 cm water filter and a red filter (26-4390, Ealing). From the emission spectrum monitored with a monochromator (Instruments S.A., Inc., Jobin Yvon Division, Metuchen, NJ) the fluence rate over the absorption peak of the photoactive monomeric dye ($\lambda_{max} \pm 20$ nm) was calculated and set at 100 W/m^2 for a final dose of 24 kJ/m^2. Experiments were repeated three times using three dishes per concentration point.

Quantum yield of singlet oxygen. Singlet oxygen quantum yields were determined for the fractionated product and AlOH-PcS$_4$ (Porphyrin Products) in deuterated methanol using the infrared luminescence technique (Rodgers and Snowden, 1982). Excitation of the soluton was by a Q switched, frequency doubled ruby laser (347 nm). Hematoporphyrin hydrochloride (Porphyrin Products) was used as standard, the absolute singlet oxygen quantum yield for this compound having been determined previously by the time resolved thermal lensing technique (Redmond and Braslavsky, 1988).

RESULTS

Partition coefficient data (Table 1) show that decreasing the number of sulfonate groups and

Table 1. Properties of differently substituted AlOH-PcSP and AlOH-PcS

Photosensitizer	Partition coefficient* (± SD)	Quantum yield of singlet oxygen†	Cellular uptake‡ (μM) (± SD)	LD$_{90}$ extracellular§ (μM)	Relative quantum efficiency of cell killing‖
AlOH-PcS$_{3.7}$P$_{0.1}$	<0.01	0.35	1.6 (0.2)	>50	—
AlOH-PcS$_{3.2}$P$_{0.4}$	0.19 (0.01)	0.38	31.0 (1.3)	0.96	0.86
AlOH-PcS$_{2.4}$P$_{1.2}$	1.12 (0.08)	0.36	82.4 (8.2)	0.28	1.09
AlOH-PcS$_4$	<0.01	0.36	—	>50	—
AlOH-PcS$_2$	0.44 (0.03)	—	14.9 (1.7)	1.7	1.0

*Dyes (25–300 nM) were partitioned between n-octanol and 100 mM Tris buffer pH 7.4.
†Quantum yields of singlet oxygen were obtained relative to hematoporphyrin dihydrochloride (ϕ_Δ = 0.64) using the IR luminescence technique.
‡Cellular uptake was measured after incubating Chinese hamster cells (V-79) with 10 μM of dyes in 1% serum media during 1 h.
§The extracellular LD$_{90}$ against V-79 cells were calculated from the survival curves presented in Fig. 2.
‖The relative quantum efficiency of cell killing was calculated from the LD$_{90}$ corrected for the amount of dye in the cells, taking the value for AlOH-PcS$_2$ as unity.

increasing the number of phthalimidomethyl substituents increase the hydrophobicity of the dyes. Selecting the most hydrophobic of the water soluble AlOH-PcSP fractions, i.e. fraction F which contained an average of 2.4 sulfonate and 1.2 phthalimidomethyl groups per Pc molecule (AlOH-PcS$_{2.4}$P$_{1.2}$), we find that the effect of incorporation of the phthalimidomethyl group results in an increase of the partition coefficient by 2.6 times.

Comparing data for cellular uptake with partition coefficients (Table 1), a relationship between hydrophobicity and cell uptake is observed. Higher hydrophobicity of dyes correlates well with an improved cellular uptake of 60 times between AlOH-PcS$_{3.7}$P$_{0.1}$ and AlOH-PcS$_{2.4}$P$_{1.2}$. The isolated effect of phthalimidomethyl addition on disulfonated dyes can be seen from the 5.6 times higher cellular uptake of AlOH-PcS$_{2.4}$P$_{1.2}$ as compared to AlOH-PcS$_2$. Fraction D, i.e. AlOH-PcS$_{3.2}$P$_{0.4}$, was the only one which did not follow the hydrophobicity–cellular uptake relationship. The three sulfonate groups contributed to partitioning the dye more in the aqueous phase while the addition of an average of 0.4 phthalimidomethyl allowed a cellular uptake 2.1 times higher than the amphiphilic AlOH-PcS$_2$. This increase in cellular uptake corresponds with an increase in photocytotoxicity of the dyes *in vitro* (Fig. 2). AlOH-PcS$_{3.2}$P$_{0.4}$ and AlOH-PcS$_{2.4}$P$_{1.2}$ exhibited LD$_{90}$ values (extracellular) of 0.96 and 0.28 μM respectively, while AlOH-PcS$_{3.7}$P$_{0.1}$ failed to achieve 90% cell mortality even at the highest concentrations used (50 μM). The influence of the phthalimidomethyl group on photocytotoxicity was clearly indicated by comparing AlOH-PcS$_{2.4}$P$_{1.2}$ with AlOH-PcS$_2$ where inclusion of the phthalimidomethyl moiety resulted in an increased photodynamic potency of more than 6-fold (AlOH-PcS$_2$, LD$_{90}$ = 1.7 μM; Paquette *et al.*, 1988). The differing phototoxic effects do not relate to the efficiency of singlet oxygen production for the
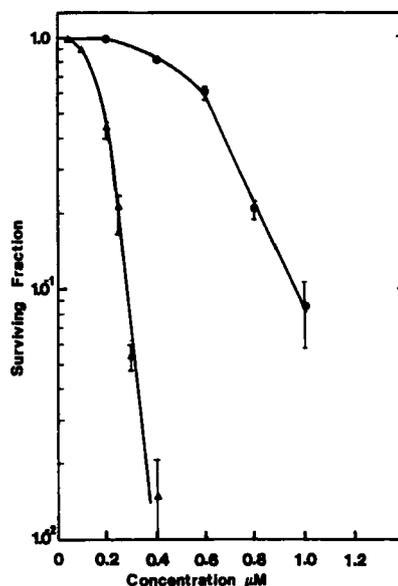

Figure 2. Survival of V-79 cells incubated for 1 h at 37°C with AlOH-PcS$_{3.2}$P$_{0.4}$ (●) and AlOH-PcS$_{2.4}$P$_{1.2}$ (▲) followed by exposure to red light. Experiments were repeated three times using three dishes per concentration points.

monomerized fractions of the various AlOH-PcSP in homogeneous solution, since all the dye preparations gave similar quantum yields (Table 1). The effect of inclusion of phthalimidomethyl groups in AlOH-PcS did correlate very well with the relative quantum efficiency of cell killing of the various dye preparations (Table 1). After expressing phototoxicity as a function of cellular uptake, AlOH-PcS$_2$ and AlOH-PcS$_{2.4}$P$_{1.2}$ exhibited the same relative efficiency, while AlOH-PcS$_{3.2}$P$_{0.4}$ was only 14% less phototoxic.

DISCUSSION

These results show clearly that the higher cellular uptake of differently substituted AlOH-PcSP *in*

vitro is associated with the more hydrophobic derivatives of sulfonated phthalocyanines. Comparing dye fractions $AlOH\text{-}PcS_{2.4}P_{1.2}$ and $AlOH\text{-}PcS_2$, the fraction substituted with phthalimidomethyl groups partitioned more in the *n*-octanol phase, and exhibited both increased cellular uptake and photocytotoxicity by about a factor of six without showing significant differences in the quantum yield of singlet oxygen. Thus, the strategy of limiting the degree of sulfonation to obtain an amphiphilic photosensitizer, whilst increasing the number of phthalimidomethyl substituents to increase hydrophobicity, and hence membrane penetration, results in an increased photodynamic effect.

It has been reported that amphiphilic dyes interact with the lipid portion of plasma membranes (Chatelier *et al.*, 1985), thereby facilitating transport of these molecules into the cell (Paquette *et al.*, 1988). This same amphiphilic property allows more efficient binding to low density lipoprotein (LDL), which has been implicated in the transport of porphyrin based sensitizers to tumorous tissue (Kessel *et al.*, 1987). Tetraphenylporphine monosulfonate, and the more hydrophobic of the disulfonated isomers bound to a greater extent with LDL, and were preferentially localized in neoplastic cells. In contrast, the tetra-, tri- and less hydrophobic disulfonated isomer were associated more with the albumin fraction of the serum proteins and distributed mainly in the stroma of the tumor. Although little additional data has been published to corroborate these results, with regard to phthalocyanines, it might be expected on the basis of sensitizer structure, that both tetraphenylporphine and Pc's, with the same degree of sulfonation, would exhibit similar affinities for the different serum lipoproteins and thence be distributed in a like manner. Thus the probability of a direct tumor response should be greater if the hydrophobicity of the molecule is increased whilst retaining water solubility.

Addition of hydrophobic phthalimidomethyl groups to AlOH-PcS results in a dramatic increase in cell uptake. A one hour incubation period of V-79 cells with 10 μM $Al\text{-}OH\text{-}PcS_{2.4}P_{1.2}$ did result in an 8-fold higher intracellular as compared to extracellular, dye concentration (Table 1). The phthalimidomethyl group did not however, appear to modify the intracellular distribution of the dye as the relative quantum efficiency of cell killing of $AlOH\text{-}PcS_{2.4}P_{1.2}$ and $AlOH\text{-}PcS_2$ were similar.

Kessel (1986) and Moan *et al.* (1983) have reported that plasma membrane is an important target following *in vitro* PDT. This assumption on the nature of the intracellular target is supported by a correlation between plasma membrane damage and loss of cell viability after a short incubation period with HpD. Our results with both $AlOH\text{-}PcS_{2.4}P_{1.2}$ and $AlOH\text{-}PcS_{3.2}P_{0.4}$ were consistent with this assumption. A more hydrophobic amphiphilic dye, such as $AlOH\text{-}PcS_{2.4}P_{1.2}$, should interact with

the lipid portion of plasma membrane (Chatelier *et al.*, 1985). It therefore seems logical to obtain a higher membrane interaction, and thus optimal cell uptake, by increasing the hydrophobicity of an amphiphilic dye. In the case of $AlOH\text{-}PcS_{3.2}P_{0.4}$, the relative yield of cell killing was only 14% lower than that observed for $AlOH\text{-}PcS_2$. This result seemed surprising, since it has been previously established that phthalocyanines substituted with three sulfonate groups interact poorly *in vitro* with the plasma membrane of V-79 cells, resulting, even after 24 h incubation, in little cell uptake and photocytotoxicity (Brasseur *et al.*, 1987; Paquette *et al.*, 1988). The relatively low partition coefficient of $AlOH\text{-}PcS_{3.2}P_{0.4}$, as compared to the amphiphilic $AlOH\text{-}PcS_2$, also suggests a weak interaction with the membrane, however, both the cellular uptake and phototoxic effect were improved. Thus, these results indicate that the addition of phthalimidomethyl groups on $AlOH\text{-}PcS_{3.2}P_{0.4}$ compensates for the adverse hydrophilic effect of the sulfonate substituents, and induces phototoxicity by permitting interaction with the plasma membrane, subsequently mediating photoinduced damage to the tumor cell.

In summary, these *in vitro* data with V-79 cells confirm the correlation between hydrophobicity, cellular uptake and cell killing of the photosensitizer, and demonstrate the relevance of introducing phthalimidomethyl groups into amphiphilic sulfonated phthalocyanines to improve cellular uptake and photodynamic action. The involvement of this correlation, with regard to both serum distribution of dye and tumor response *in vivo*, is currently being investigated in our laboratory.

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