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BIOLOGICAL ACTIVITIES OF PHTHALOCYANINES—VIII. CELLULAR DISTRIBUTION IN V-79 CHINESE HAMSTER CELLS AND PHOTOTOXICITY OF SELECTIVELY SULFONATED ALUMINUM PHTHALOCYANINES

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Abstract—Water soluble chloro aluminum phthalocyanines sulfonated to different degrees are studied for phototoxicity and cellular distribution in V-79 Chinese hamster cells. The more hydrophobic disulfonated dyes, with sulfonate substituents on adjacent benzyl groups of the phthalocyanine ring structure, exhibited the best cell penetrating properties and the highest phototoxicity. Fluorescence microscopy revealed that the dye was uniformly distributed in the cytoplasm but absent in the nucleus. The greater cell membrane penetrating properties of the lower as compared to the higher sulfonated dyes are attributed to the amphiphilic nature of the former.

INTRODUCTION

Photodynamic therapy (PDT)† of cancer, using porphyrin derivatives as photosensitizers, is undergoing extensive clinical trials and several promising results have been reported (Dougherty, 1987). Advantages of this procedure as compared to conventional cancer treatment modalities include selectivity and low systemic toxicity. Further improvement of PDT can be envisaged through the use of more efficient photosensitizers. Among the various dyes proposed to this end, phthalocyanines (PC) and their water soluble sulfonated derivatives (SPC) have received increasing attention. Appropriate properties include their strong absorption of tissue penetrating red light, apparent absence of toxicity, well defined chemistry and photochemical stability (for recent reviews, see Spikes, 1986; Ben-Hur *et al.*, 1987). The photochemical and photophysical properties of various PC dyes has been studied (Darwent *et al.*, 1982; Harriman and Richoux, 1980; Langlois *et al.*, 1986; Spikes and Bommer, 1986; Wagner *et al.*, 1987) and both the *in vitro* and *in vivo* phototoxicity of PC dyes has been demonstrated (Brasseur *et al.*, 1985, 1987; Ben-Hur and Rosenthal, 1985, 1986; Chan *et al.*, 1986; Bown *et al.*, 1986; Selman *et al.*, 1986).

We have recently observed, using a series of gallium sulfophthalocyanines (GaCl-SPC) that varying the degree of sulfonation does not directly alter the kinetics of $^1O_2$ production from the excited dye but rather these dyes have different tendencies to aggregate in a particular solvent (Wagner *et al.*, 1987). Lower degrees of dye sulfonation results in a shift in the chemical equilibrium away from the productive monomeric state and consequently a reduced photochemical yield. In contrast, the lower sulfonated hydrophobic GaCl-SPC dyes exhibited the highest photocytotoxicity (Brasseur *et al.*, 1987a, b). We now report on the cellular uptake and distribution of AlCl-PC dyes sulfonated to different degrees in an attempt to correlate hydrophobicity with cell membrane permeability and photocytotoxicity. We selected the aluminum complex for these studies in view of the strong fluorescence properties of this dye.

MATERIALS AND METHODS

Aluminum sulfophthalocyanines. AlCl-PC was purchased from Eastman Kodak, Rochester, NY and sulfonated with fuming (30% SO_3) sulfuric acid (Linstead and Weiss, 1950) to yield an isomeric mixture of mono- to tetrasulfonated AlCl-SPC. The mixture was fractionated by preparative, medium pressure chromatography on a 30 cm long by 2 cm ID glass column packed with C-18 reverse phase (25–40 μm) using a linear gradient of 0 to 95% MeOH in 10m*M* phosphate buffer (pH 5). Fractions were analyzed by HPLC on a C-18 reverse phase column (ODS-2 spherisorb, 5 μm, 25 cm long by 0.94 cm ID from CSC, Montreal). The column was operated at 2 mℓ min^{-1} with a linear gradient (55 min) from 0 to 95% MeOH in 10 m*M* sodium phosphate buffer followed by an isocratic mixture (15 min) of 95% MeOH in buffer. Eluting SPC were detected at 650 nm. In this manner four main fractions were collected (Fig. 1) consisting of (A) a mixture of 40% trisulfonated and 60% tetrasulfonated AlCl-SPC (S/PC ratio 3.6), (B) 30% disulfonated (mixture of least hydrophobic isomers) and 70% trisulfonated AlCl-SPC (S/PC ratio 2.7), (C) a mixture of 90% disulfonated AlCl-SPC (the most hydrophobic isomer) and 10% disulfonated AlCl-SPC (the more hydrophylic isomers) (S/PC ratio 2.0) and (D) monosulfonated AlCl-MSPC (S/PC ratio 1.0). The S/PC ratio was determined by an oxidative degradation assay (Ali *et al.*, unpublished results). Briefly a small sample (1 mg) of AlCl-SPC is oxidized in a minimal volume of concentrated HNO_3 to give the corresponding

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†*Abbreviations*: PC, phthalocyanine; PDT, photodynamic therapy; PP, protoporphyrin; SPC, sulfophthalocyanine; S/PC ratio, number of sulfonates per PC molecule.

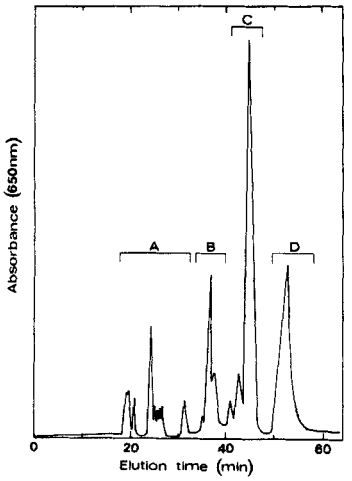

Figure 1. Tracing of reverse phase HPLC elution pattern of AlCl-SPC prepared by sulfonation of AlCl-PC with oleum. The material was separated in four main fraction A–D with average S/PC ratios of 3.6 (A), 2.7 (B), 2.0 (C) and 1.0 (D).

sulfophthalimide and phthalimide fragments which are quantified by their absorption at 215 nm after reverse phase HPLC. Retention of aluminum in the PC ring system during the synthesis and purification of the differently sulfonated AlCl-SPC was confirmed by their absorption spectra in 100% MeOH: λ_{max} 674 nm, ϵ 19 × 10^4 M^{-1} cm^{-1}. The AlCl-MSPC, which is poorly water soluble and precipitated upon purification, was not included in the following experiments.

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). Cells were maintained in growth medium (MEM) supplemented with 15% fetal bovine serum, 1% L-glutamine and 1% vitamins (Flow Lab.). Cells in log phase were plated in 60 mm petri dishes with growth medium and incubated for 3 h at 37°C in 5% CO_2 to allow cell attachment. The number of cells plated was adjusted such as to yield 200 colonies after treatment. The cells were rinsed with PBS and incubated for 1 h in the dark at 37°C with 1 mℓ of medium containing 1% serum and 0.25–50 μ*M* dye. After removal of the dye and washing with PBS, the cells were exposed at room temperature for 4 min to red light. The fluence rate over the absorption peak of the photoactive monomeric dye (654–694 nm) was 101 W m^{-2}. After phototreatment, the cells were re-fed with growth medium and incubated at 37°C in 5% CO_2 for 6–7 days. Experiments were repeated 3 to 4 times using three dishes per concentration point. In a second series of experiments the dye incubation time was varied from 0.25–24 h using 3mℓ of OPTI-MEM medium (Gibco) supplemented with 1% serum, 1% L-glutamine, 1% vitamins and 2 μ*M* dye.

Fluorescence microscopy. Experiments were conducted as described by Chan *et al.* (1986) and Austen *et al.* (1978) with minor modifications. Briefly, about 10^6 V-79 cells were plated in 60 mm petri dishes and incubated with complete growth medium for 10 h at 37°C in a CO_2 incubator. Cells were then rinsed twice with PBS and incubated for 0.25–24 h in the dark with 0.01–100 μ*M* in OPTI-MEM medium containing 1% serum. In the case of the disulfonated AlCl-SPC (S/PC ratio 2.0) 50 μ*M* gave optimal cell fluorescence after 24 h incubation. Accordingly, this concentration was used for further studies. After removal of the dye solution, cells were rinsed twice with PBS, fixed with 10% formaline for 30 s and again rinsed with PBS. Cells were placed with PBS in a 24 × 30 mm lamela and observed through a fluorescence microscope (Leitz Wetzlar, 540 × magnification) fitted with an excitation filter (1 mm UV UG 1, 3 mm Blue B G 38 and a diffusor) and a K530 cutoff filter. Photographs were obtained on a TRI-X PAN 400 ASA Kodak film. Fluorescence intensities at 690 nm of the various dye solutions over a concentration range of 1.25–50μ*M* in MeOH were measured with a Carl Zeiss spectrofluorometer using 370 nm excitation light and a cuvette with a 1 cm pathlength.

RESULTS

Control V-79 cells without added dye showed no effect on growth at the highest fluence of red light used in these studies (27 kJ/m^2). Cells incubated with AlCl-SPC (S/PC ratio 2 to 4) up to 50 μ*M* for 1 h without subsequent exposure to light showed near 100% cell survival. Increasing the incubation time to 24 h with 2 μ*M* of the various dyes without red light exposure also showed no significant mortality.

Effect of the degree of sulfonation of AlCl-SPC on phototoxicity

The effect of AlCl-SPC sulfonated to different degrees (average S/PC ratios 2.0, 2.7 and 3.6) on V-79 cell survival after a 1 h incubation with the dye followed by a 4 min exposure to red light is presented in Fig. 2. The concentration of the various dyes required to achieve 90% cell mortality (LD_{90}) increases drastically with increasing S/PC ratio of the AlCl-SPC preparation. Thus, AlCl-SPC with an average S/PC ratio of 2.0 is the most efficient photosensitizer for cell killing with a LD_{90} of 1.7

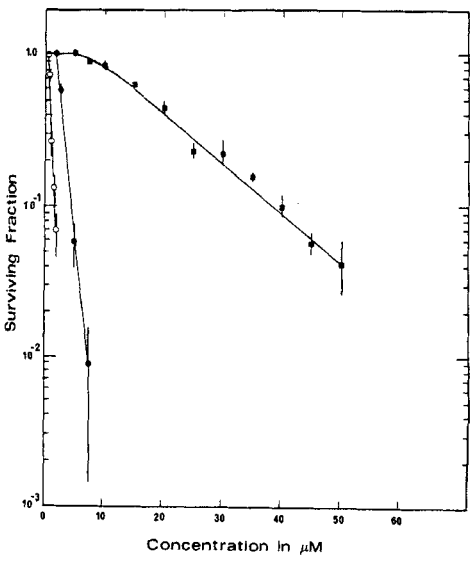

Figure 2. Survival of V-79 cells as a function of AlCl-SPC concentration after exposure for 4 min to red light (λ > 590 nm, 101 W^{-2}). The symbols correspond to AlCl-SPC preparations with average S/PC ratios of 2.0 (○), 2.7 (●) and 3.6 (■).

μM (Fig. 2). Increased percentages of tri- and tetra-sulfonated dye in the AlCl-SPC mixture results in a higher LD_{90} of 4.5 μM and 38.3 μM for preparations with average S/PC ratios of 2.7 and 3.6 respectively. Accordingly, under the above experimental conditions, the lower sulfonated fraction (S/PC ratio 2.0) is 25 times more efficient in photoinactivation of V-79 cells than the higher sulfonated fraction (S/PC ratio 3.6). Since the former is substantially enriched in hydrophobic disulfonated dyes (Fig. 1), these data suggest that varying photocytotoxicity of AlCl-SPC mainly reflects the different percentage of hydrophobic disulfonated AlCl-SPC in the preparations.

The effect of the incubation time

The effect of the degree of sulfonation on the relative phototoxicity of the AlCl-SPC preparation is even more pronounced upon varying the incubation time of the cells with the various dye preparations (2 μM), followed by a 4 min exposure to red light (Fig. 3). Under these conditions, the higher sulfonated preparation (S/PC ratio 3.6) showed no significant effect, even after prolonged incubation times of 24 h. The disulfonated fraction (S/PC ratio 2.0) required a 0.6 h incubation period to induce a 90% mortality (LT_{90} = 0.6 h), whereas the preparation with a S/PC ratio of 2.7 required a 17 fold longer period to induce the same effect (LT_{90} = 10.4 h).

Fluorescence microscopy

Cell uptake and distribution was studied by fluorescence miscroscopy using λ=370 nm excitation light. Since the emission spectrum (λ_{max} = 690 nm) overlaps the absorption spectrum (λ_{max} = 674 nm) of the AlCl-SPC we may expect self-quenching of the emitted light. Accordingly we compared the intensity of the fluorescence at λ = 690 nm for the three AlCl-SPC preparations over a concentration range of 1.25 to 50 μM in 100% MeOH (Fig. 4). Maximum intensity of the fluorescence is observed between 2.5 and 5 μM. Due to self-quenching, fluorescence is not proportional to the dye concentration. However, all three dye preparations follow a similar absorption pattern, thus permitting at least a qualitative comparison of their cellular distribution by fluorescence microscopy.

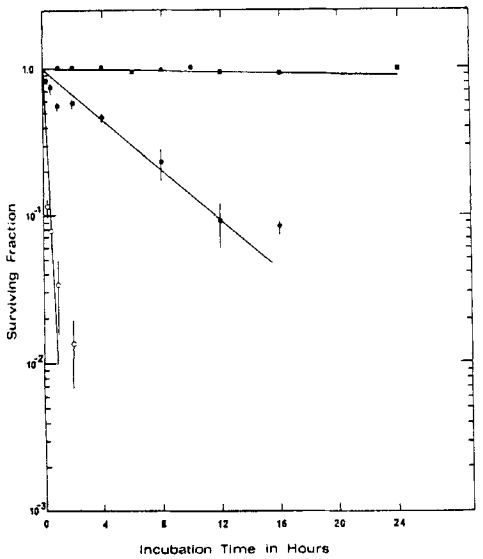

Figure 3. Survival of V-79 cells incubated with 2 μM AlCl-SPC as a function of the incubation time. The symbols correspond to AlCl-SPC preparations with average S/PC ratios of 2.0 (○), 2.7 (●), and 3.6 (■).

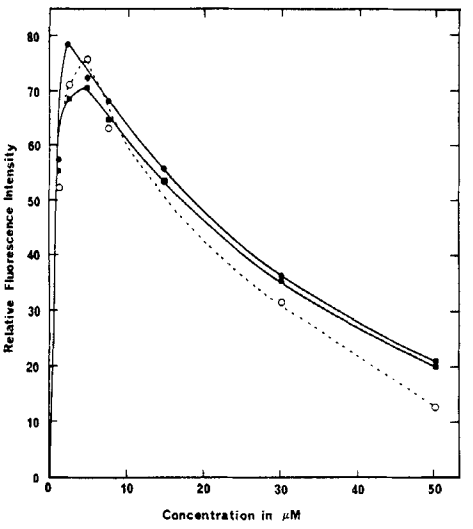

Figure 4. Relative fluorescence intensity measured in MeOH at λ = 690 nm (λ_{excit} = 370 nm) of AlCl-SPC sulfonated to different degrees. Average S/PC ratios of the dye preparations: 2.0 (○), 2.7 (●) and 3.6 (■).

As may be seen from the fluorescence photographs of V-79 cells, obtained after a 24 h incubation period with 50 μM dye, very little dye uptake is observed with the highest sulfonated AlCl-SPC (Fig. 5C). In contrast, the lower sulfonated fractions gave uniformly fluorescence in the cell cytoplasm, with the disulfonated fraction (S/PC ratio 2.0) showing the highest intensity (Fig. 5A) and most rapid (not shown) cell uptake. We did not observe any fluorescence in the cell nucleus with any of the AlCl-SPC preparations.

DISCUSSION

The degree of sulfonation of phthalocyanines affects a number of properties of the dyes of importance for their potential use in PDT, they include water solubility, tumor localization, cell membrane uptake and penetration, tendency to aggregate and capacity to generate singlet oxygen. We have previously shown that these parameters are interrelated

and that they may either enhance or neutralize each other in terms of the phototherapeutic efficiency of the SPC (Brasseur *et al.*, 1987; Wagner *et al.*, 1987). In this study we have compared the effect of the degree of sulfonation of AlCl-SPC on its photocytotoxicity and we have attempted to correlate this effect with the cell membrane permeability and intracellular action sites of the various SPC. Two series of experiments were conducted to determine the relative phototoxicity of the dyes. Varying concentrations of the AlCl-SPC (LD_{90}) and varying incubation times with a predetermined concentration of the dye (LT_{90}) indicated that the lowest sulfonated preparation with an average S/PC ratio of 2.0 exhibited the highest photocytotoxicity (LD_{90} = 1.7 μM, LT_{90} = 0.6 h). This correlates well with the high percentage of disulfonated dye (90%) in this preparation. Furthermore, since the dye was purified by fractionation on a reverse phase HPLC column, this preparation is particularly enriched in the most hydrophobic disulfonated AlCl-SPC isomer which, in analogy with observations on isomeric disulfonated tetraphenylporphyne derivatives (Kessel, 1987), most likely consists of SPC with sulfonate substituents on adjacent benzene rings (Fig. 6). The fraction with a S/PC ratio of 2.7 is substantially less active (LD_{90} = 4.5 μM and LT_{90} = 10.4 h). Although this fraction contains 30% of disulfonated dye, it is enriched in the least hydrophobic isomers (Fig. 1), presumably consisting of SPC with sulfonate substituents located at opposite positions of the PC molecule (Fig. 6). The mixed tri- and tetrasulfonated preparation (S/PC ratio 3.6) showed very little photocytotoxicity (LD_{90} = 38.3 μM).

Fluorescence microscopy of V-79 cells treated with the various dye preparations suggests that the differences in photocytotoxicity result from differences in their cell membrane permeability. The more hydrophobic disulfonated AlCl-SPC showed good cell uptake with uniform fluorescence in the cytoplasm and high photocytotoxicity, whereas the least hydrophobic tetra- and trisulfonated AlCl-SPC showed no cell penetration and insignificant photocytotoxicity. It thus appears that the observed photocytotoxicity is mainly due to the disulfonated dye, and particularly to the most hydrophobic of the various disulfonated constitutional isomers. In analogy with observations on disulfonated phenylporphyrins, (Kessel, 1987) such isomers are depicted with sulfonate substituents on adjacent benzyl rings of the PC molecule (Fig. 6). Using highly purified disulfonated GaCl-SPC isomers, we recently observed a similar structure-photocytotoxicity pattern for the gallium complex. As with the aluminum complex, the least hydrophobic isomer with sulfonate groups at opposite positions of the PC molecule was completely inactive whereas the PC molecule substituted with sulfonate groups at adjacent benzyl rings was highly photocytotoxic (Brasseur *et al.*, 1987b). Protein-free lipid bilayer

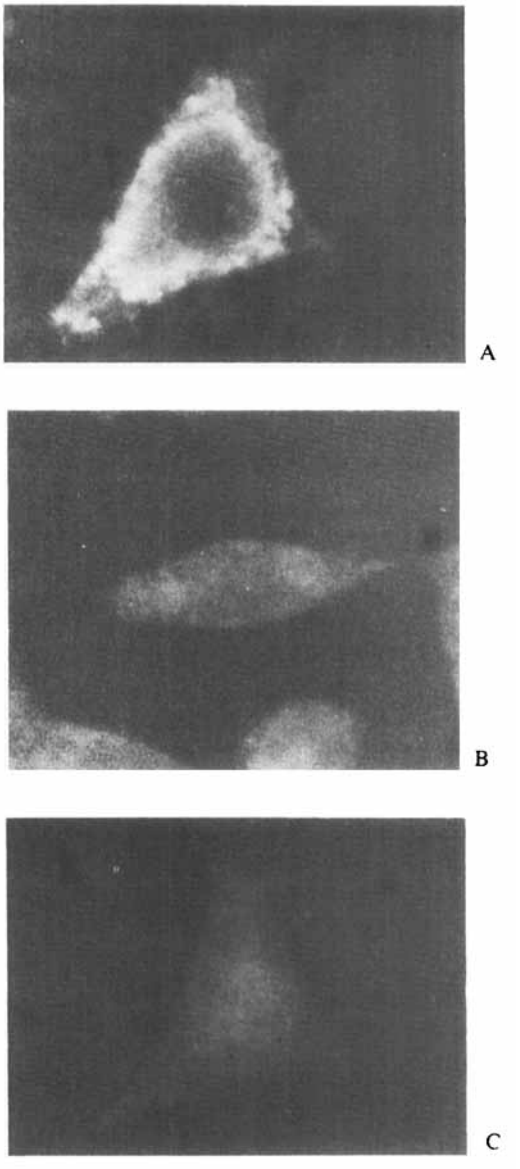

Figure 5. Fluorescence microscopy (λ_{excit} = 370 nm) of V-79 cells incubated for 24 h with 50 μM of AlCl-SPC sulfonated to different degree. Average S/PC ratios of the dye preparations: (A) 2.0, (B) 2.7 and (C) 3.6.

are impermeable to charged molecules and a membrane transfer molecule is required to facilitate transport of charged species into the cell (Alberts *et al.*, 1983). Membrane interaction of charged molecules has been studied by Chatelier (1985) using protoporphyrin (PP) and artificial lipid bilayers. Their work suggested that PP behaves like a typical amphiphile with charged substituent located at the membrane/buffer interface and the non-polar portion of the molecule in contact with the hydrophobic lipid chains. A similar mechanism could explain the cellular uptake of our disulfonated SPC dyes (Fig. 6). Such a dye-membrane interaction would allow the charged SPC to bind to membrane transport

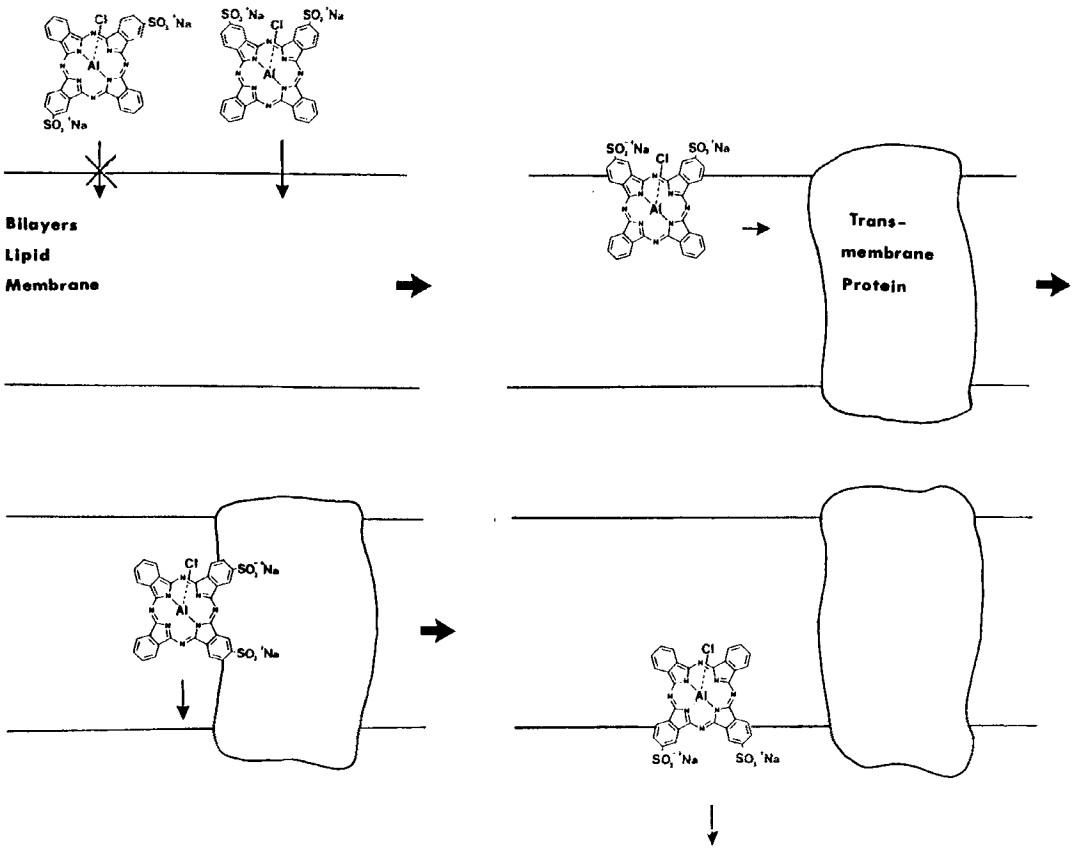

Figure 6. Proposed mechanism for the membrane transport of disulfonated AlCl-SPC. Only amphiphilic isomers, with sulfonate substituents located at adjacent benzyl rings of the PC backbone, are capable to interact with hydrophobic membrane lipids thus allowing the charged dye to bind to a membrane transport protein and to enter the cell.

protein and to enter the cell cytoplasm. Only amphiphilic isomers, with sulfonate substituents located at adjacent benzyl rings of the PC backbone, are capable to cross the cell membrane in this model. Disulfonated dyes with substituents on opposite positions of the PC structure, as well as tri- and tetrasulfonated derivatives lack the essential amphiphilic nature thus explaining their poor cell penetrating properties and low photocytotoxicity. Finally, the absence of AlCl-SPC in the nucleus may be explained by the low dye concentration in the cytoplasm. Ben-Hur and colleagues (1986) estimated an uptake of 0.4 n*M* per 10^6 cells after a 16 h incubation period of V-79 cells with 25 μM of AlCl-SPC. Accordingly, within the cell the concentration gradient between the cytoplasm and the nucleus may be too low to result in detectable dye concentrations in the latter. It should be noted however that DNA damage induced by SPC and red light has been observed (Ben-Hur *et al.*, 1987; Hunting *et al.*, 1987) suggesting that reactive photogenerated species are capable to diffuse into the nucleus or alternatively that low dye levels are present in the nucleus.

In summary, we have shown that water soluble disulfonated AlCl-SPC, particularly the most hydrophobic constitutional isomers, can diffuse readily through a cellular membrane. The capacity of the dye to enter the cytoplasm correlates well with their photocytotoxicity suggesting that such disulfonated SPC are promising candidates for *in vivo* PDT. Water soluble tri- and tetrasulfonated SPC have poor cell penetrating and photocytotoxic properties whereas clinical application of the mono- and non-sulfonated dyes may be limited due to their poor water solubility. Finally, our findings underline the importance of the use of well characterized SPC preparations in comparative studies of different metallo complexes for their potential use in PDT.

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