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BIOLOGICAL ACTIVITIES OF PHTHALOCYANINES—XI. PHOTOTOXICITY OF SULFONATED ALUMINUM NAPHTHALOCYANINES TOWARDS V-79 CHINESE HAMSTER CELLS

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Abstract—The phototoxicity of sulfonated aluminum naphthalocyanines towards V-79 Chinese hamster cells is investigated. The disulfonated naphthalocyanine exhibits similar photostability, but better cell penetrating properties than the tetrasulfonated dyes. The capacity of the naphthalocyanines to generate singlet oxygen is comparable to that of the corresponding phthalocyanines. However, in contrast to the phthalocyanine dyes, the sulfonated aluminum naphthalocyanines show very little phototoxicity towards the V-79 cells, suggesting close association with non-vital cell constituents or extensive formation of photoinactive adducts and aggregates.

INTRODUCTION

Photodynamic therapy (PDT)† has been proposed as an alternative treatment modality to complement conventional protocols in the management of malignant tumors. Specific accumulation of non-toxic dyes and localized application of therapeutic red light allows for a higher selectivity of PDT protocols as compared to conventional therapies of tumors. The photosensitizer preparation currently used in clinical trials of PDT consists of a mixture of hematoporphyrin derivatives (HPD); a preparation enriched in the active components is marketed as Photofrin II™. The effectiveness of PDT could be enhanced by the use of sensitizers which are more selectively retained by tumors and which absorb more strongly towards the red end of the light spectum. To date, using the latter approach, numerous porphin analogs with red-shifted absorption maxima have been evaluated as possible second generation sensitizers for PDT (for a review see van Lier, 1988). Among them, naphthalocyanines (Nc) (Fig. 1) are of particular interest, distinguishing themselves by a strong absorption band at about 760 nm which corresponds to a further 100 nm red-shift as compared to the structural analogous phthalocyanines (Pc) (Fig. 2). The latter dyes have been studied extensively for their potential use in PDT (for reviews see Spikes, 1986; Ben-Hur, 1987; van Lier *et al.*, 1988; van Lier and Spikes, 1989). At 750–800 nm light penetration of tissue is two fold better than at the highest absorption band of hematoporphyrin derivatives, e.g. 630 nm (Anderson and Parrish, 1982). Although the synthesis of Nc was reported by Bradbrook and Linstead as long ago as 1936, this dye was only recently suggested for use in PDT (Spikes, 1986). Some photophysical and photochemical studies on Nc's and their water-soluble sulfonated derivatives (NcS), and particularly their capacity to generate $^1O_2$, suggest their potential value for PDT (McCubben and Phillips, 1986; Firey and Rodgers, 1987; Firey *et al.*, 1988). To further investigate the potential of Nc as a photosensitizers for PDT, we evaluated the photodynamic properties of aluminum naphthalocyanines, sulfonated to different degrees, using the following parameters: (a) capacity to photooxidize tryptophan in homogeneous solution, (b) uptake by V-79 Chinese hamster cells *in vitro*, (c) photostability in V-79 cells and (d) toxicity and photocytotoxicity towards V-79 cells.

MATERIALS AND METHODS

Sulfonated aluminum phthalocyanines (Al–PcS_{1-4}) were prepared as previously described (Ali *et al.*, 1988). The corresponding naphthalocyanines were prepared in a similar manner. The spectral properties of the monomeric dyes, i.e. in 100% MeOH, were: Al–PcS, λ_{max} 674 nm, ϵ 19×10^4 M^{-1} cm^{-1}; Al–NcS, λ_{max} 771 nm, ϵ 16×10^4 M^{-1} cm^{-1}.

Mono- to tetrasulfonated aluminum naphthalocyanine (Al–NcS). Chloroaluminum naphthalocyanine (AlCl–Nc) was prepared by condensation of 2,3-dicyanonaphthalene and $AlCl_3$ (anhydrous) at 270–280°C for 1 h. The sulfonation procedure was adapted from Linstead and Weiss (1950). Briefly, AlCl–Nc (200 mg) was dissolved in 5 mℓ oleum (fuming H_2SO_4 containing 15% free SO_3) at 50°C with stirring. The mixture was kept at this temperature for 45 min whereafter it was poured onto crushed ice. The resulting dark green precipitate was filtered and washed with 1N HCl (10 mℓ), redissolved in 1N NaOH (15 mℓ)

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†*Abbreviations*: DMF, *N*,*N*-dimethylformamide; HPPI, 3a-hydroperoxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3b] indole-2-carboxylic acid; Nc, naphthalocyanine; NcS_{1-4} mono- to tetrasulfonaphthalocyanine; PBS, phosphate buffered saline; Pc, phthalocyanine; PcS_{1-4}, mono- to tetrasulfophthalocyanine; PDT, photodynamic therapy; Φ, molecules formed per absorbed photon.

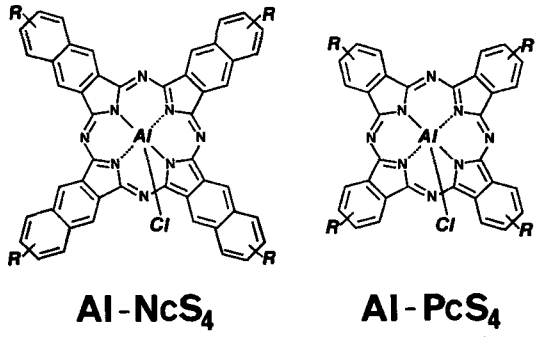

Figure 1. Chemical structure of sulfonated aluminium naphthalocyanine (Al–NcS) and sulfonated aluminum phthalocyanine (Al–PcS). R = SO_3^- or H.

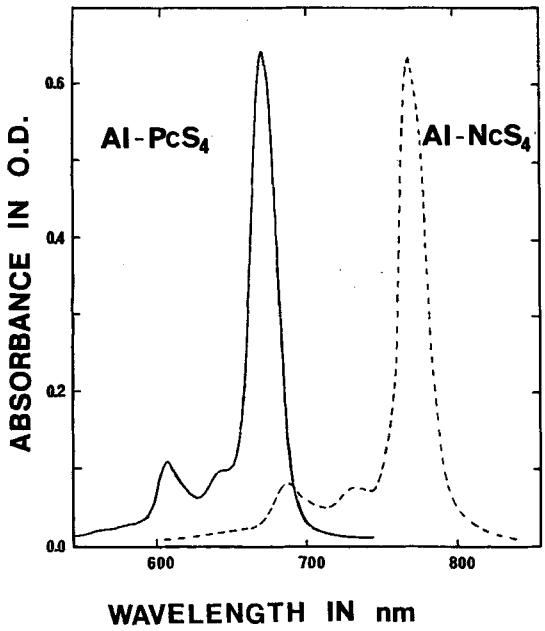

Figure 2. Absorption spectra of Al–PcS_4 and Al–NcS_4 (3 μM) in MeOH/H_2O (95:5) in a 1 cm path quartz cuvette.

and filtered again in order to remove insoluble impurities, including non-sulfonated naphthalocyanine. The filtrate was neutralized with 1N HCl and analyzed by HPLC, revealing the presence of tetra-(major) and trisulfonated (minor) products. In order to obtain mono- and disulfonated products, AlCl-Nc (100 mg) was dissolved in 1.5 mℓ of oleum and 1.5 mℓ H_2SO_4 (sp. gr. 1.84). The reaction mixture was kept at 75–80°C for 1–4 h (depending on the degree of sulfonation required), poured onto crushed ice and worked up in the usual manner.

Mixed Al–NcS (100 mg) in 100 mℓ of water were fractionated by chromatography on a 30 cm long × 2 cm i.d. glass column packed with C-18 reverse phase, particle size 25–40 μm (Macherey-Nagel, Düren, Germany). Elution was carried out with a stepwise gradient of 0–100% MeOH in 10 mM sodium phosphate buffer, pH 5.0. Al–NcS sulfonated to different degrees were collected, concentrated under vacuum, adsorbed on a small amount of reverse phase (C-18) packing, washed with water to remove salts and eluted with MeOH (25–70%) in water. The composition of the various purified Al–NcS_{1-4} fractions was determined by HPLC in an identical manner as described for the analogous Al–PcS_{1-4} (Ali *et al.*, 1988), except that NcS were detected by their absorption at 700 nm. Briefly, this system consists of a 25 cm (0.94 cm i.d.) reverse phase column packed with ODS-2 spherisorb, 5 μm (CSC, Montreal) operated at 2 mℓ min^{-1} with a linear gradient (55 min) from 0 to 95% MeOH in 10 mM sodium phosphate buffer, pH 5. Retention times of the Al–NcS_{1-4} were similar to those observed for the corresponding Al–PcS_{1-4} and ranged from 20 to 25 min for the tetra-, 25 to 35 min for the tri-, 35–55 min for the di-, and 55 to 65 min for the monosulfonated derivatives.

Determination of the degree of sulfonation. In addition to HPLC analysis, the average degree of sulfonation of the various Al–NcS fractions was determined *via* quantification of the ratios between sulfonated and nonsulfonated naphthalimide fragments derived from the oxidative degradation of the Nc macrocycle (Ali *et al.*, 1988). For this assay about 1 mg of Al–NcS was dissolved in 1.8 mℓ of DMF followed by the addition of 0.2 mℓ HNO_3 (conc.). The solution was heated (50–90°C) until the characteristic blue-green color disappeared and then neutralized with 1N NaOH. An alternative degradation procedure which results in the formation of fewer secondary products and which is applicable to both PcS and NcS dyes, involves the use of ammonium cerium(IV) nitrate as an oxidizing agent. In the latter case, approx. 1 mg of the dye was dissolved in a few mℓ of MeOH/H_2O (1:1) followed by the addition of a few mg of the cerium salt. The mixture was heated (50–90°C) until the color disappeared. Product analysis after both degradation procedures was performed on a reverse phase HPLC column (25 cm long × 0.94 cm i.d.) packed with ODS-2 on 5 μm spherisorb (CSC, Montreal), operated at 2 mℓ/min with 0.1% trifluoracetic acid in water (5 min) followed by a 30 min linear gradient from this solvent to 100% MeOH. Phthalimide and naphthalimide products are well separated in this system and quantified by manual integration of their absorption peaks at 215 nm. Retention times include 3-sulfophthalimide 15 min, 4-sulfophthalimide 16 min, phthalimide 28 min, sulfonaphthalimide 22 min and naphthalimide 32 min.

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 10% 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 10–100 μM dye. After removal of the dye and washing with PBS, the cells were exposed at room temperature for 4 min to red light from a 500 W tungsten/halogen lamp equipped with a 10 cm water filter and a red filter (LL-700-S-J579, Corion). The emission spectrum is presented in Fig. 3. The fluence over the absorption peak of the photoactive monomeric dye (751–791 nm) was 27 kJ/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 whereafter colonies were counted. Experiments were repeated twice using four dishes per concentration point.

Cell uptake and photostability. About 4 × 10^6 Chinese hamster lung fibroblast 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, 70 μM Al–NcS in 1 ml of medium containing 1% serum was added and cells were incubated for 1 additional hour in the absence of light. After removal of the medium, cells were washed three times with PBS and irradiated 8 min with red light to receive a fluence of 72 kJ/m^2 (calculated over the 751–791 nm absorbance peak of the photoactive monomeric dye). Cells were detached with 600 $\mu\ell$ trypsin-EDTA 0.25% (4–5 min incubation) and dishes were

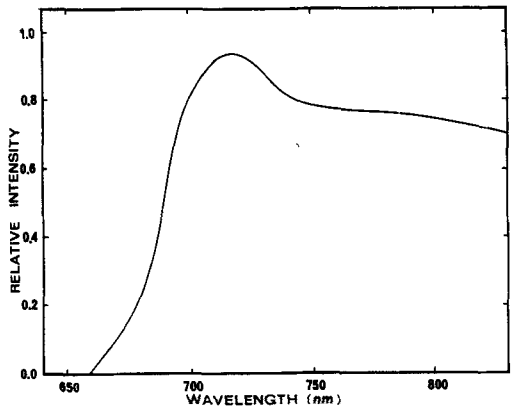

Figure 3. Emission spectrum of the light source used in the photocytotoxicity and *in vitro* photostability assays.

washed with 600 μℓ PBS. Cells were collected by centifugation in 1.5 mℓ plastic tubes (5 min, 600 *g*) and resuspended in 200 μℓ of buffer (Tris 0.2 *M*, $MgCl_2$ 10 m*M*, $CaCl_2$, 1 m*M*, 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) 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). 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. The fluorescence of the diluted cell extracts (0.5 to 1.0%) in a mixture of MeOH/H_2O (95:5) was measured after a 1 h stabilization period. Al–NcS and Al–PcS fluorescence was measured with a SLM-Aminco SPF-500C spectrofluorometer (Al–NcS: λ_{ex} = 760 nm, λ_{em} = 790 nm; Al–Pcs: λ_{ex} = 660 nm, λ_{em} = 690 nm) and concentrations were calculated by means of standard curves. The extraction efficiency was evaluated by adding a known amount of dye after the freeze-thaw treatment of the cells followed by the usual workup.

Quantification of L-*tryptophan peroxides*. The interaction of L-tryptophan with $^1O_2$ in homogeneous aqueous solution yields a pair of characteristic hydroperoxides which have been characterized as the isomeric *cis* and *trans* 3a-hydroperoxy-1,2,3,3a,8,8a-hexahydropyrrolo[2,3b]indole-2-carboxylic acids (HPPI) (Langlois *et al.*, 1986). HPPI is readily quantified by a colorimetric procedure using Fe^{2+} and xylenol orange. Fe^{2+} is oxidized to yield a Fe^{3+}-xylenol orange complex with a λ_{max} at 540 nm (Gupta, 1973). We have previously used this reaction to compare the $^1O_2$ generating capacity of differently sulfonated Ga–PcS and shown that the Φ(HPPI) is directly proportional to the Φ($^1O_2$) (Wagner *et al.*, 1987). The reagent was prepared fresh before use by mixing the following stock solutions: 10 parts of 4×10^{-4} *M* ferrous ammonium sulfate hexahydrate in 0.1 N H_2SO_4, one part of 10^{-2} *M* xylenol orange tetrasodium salt and nine parts of purified water (conductivity $< 0.25 \times 10^{-6}$ Ω^{-1} cm^{-1}).L-Tryptophan (2.0 m*M*) and the Al–PcS or Al–NcS dye (3.5 μ*M*) in MeOH/H_2O (95:5), saturated with O_2 by purging for 5 min, were irradiated for 1 h with red light. Prior to use, dye solutions were purified twice on a 1 mℓ kelex column (Sigma, dry mesh 50–100) in order to remove traces of metal ions. The irradiation apparatus consisted of a 1000 W xenon lamp collimated by a double condensing lens, a 10 cm cooled water filter, a Spectral Energy monochromator (model GM 252) mounted with a GMA 152 quartz focusing sleeve at the entrance, a red filter (Ealing 26–4390) and a lens to focus light on a quartz cuvette (1 cm path length). The amount of light absorbed was determined with an Optikon radiometer (model 88 XLC) fitted with a model 300 probe, calibrated *via* Reinekers salt actinometry. One milliliter of the irradiated sample was mixed with 2 mℓ of reagent and absorbance at 540 nm was measured after 1 h. Hydroperoxide (HPPI) formation was quantified taking ε = 2.68×10^4 M^{-1} cm^{-1} at 540 nm for the Fe^{3+}-xylenol orange complex. Values were corrected for photodegradation of the NcS and PcS dyes, which was determined from dye absorption before and after irradiation.

RESULTS AND DISCUSSION

Effect of the degree of sulfonation of Al–NcS and Al–PcS on phototoxicity

The effect of two Al–NcS fractions, sulfonated to different degrees,on V-79 cell survival is presented in Fig. 4. The Al–$NcS_{3,7}$ fraction, containing mainly tri- and tetrasulfonated dye, showed little photocytotoxicity with 73.7% cell mortality at 70 μ*M*. A substantial dark toxicity, accounting for 20% of the cell killing, was observed. The low phototoxicity of this highly sulfonated dye is in agreement with earlier observations on analogous Al–PcS preparations. Under similar experimental conditions a high LD_{90} of 38 μ*M* was reported for Al–$PcS_{3,6}$ (Paquette *et al.*, 1988) while Ga–PcS_4 showed only slight phototoxicity, even at elevated concentrations of 200 μ*M* (Brasseur *et al.*, 1987).

Surprisingly, the hydrophobic Al–$NcS_{1,9}$ fraction showed only 19% cell death at 100 μ*M*, either with or without exposure to red light. This contrasts

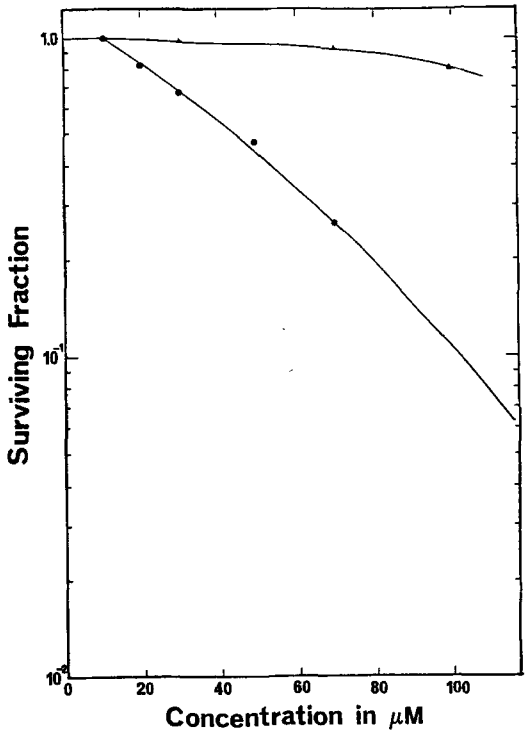

Figure 4. Survival of V-79 cells incubated for 1 h at 37°C with Al–$NcS_{1,9}$ (▲) and Al–$NcS_{3,7}$ (●) followed by exposure to red light. The colony forming assay was repeated twice using four dishes per concentration.

strikingly with our earlier observations on the analogous Al–PcS_2, which induced 90% cell death at 1.7 μM (LD_{90}) after exposure to red light, without detectable dark toxicity (Paquette *et al.*, 1988). Our failure to observe photocytotoxicity with the sulfonate naphthalocyanines could result from a number of factors, including lack of cell uptake of the dyes, high intracellular photodecomposition or inefficiency to generate phototoxic products. These possible explanations are addressed in the following studies.

Cell uptake and photostability

In view of the instability of the Nc dyes under extreme alkaline conditions, we monitored the fluorescence intensity for possible changes after the addition of NaOH during the analytical procedure. No variations were observed during the 10 min incubation period. However, cell extracts were not neutralized with HCl since this was found to lower the reproducibility of the assay. At higher dye concentrations a green precipitate occurred, but at the dye concentration used in our protocol, combined with the final 1 h incubation period in 95% MeOH, this problem was eliminated. Extraction efficiencies varied between 85 and 100%.

Cell uptake

Cell uptake of the Al–NcS and Al–PcS are presented in Table 1. Incubation conditions were adjusted to those used in the photocytotoxicity assay. It is evident from these data that the less hydrophobic Al–$NcS_{3,7}$ and Al–$PcS_{3,6}$ fractions, which contain mainly tri- and tetrasulfonated dyes, accumulate poorly in V-79 cells. Accordingly, the low level of photocytotoxicity observed with highly sulfonated Nc or Pc dyes appears to reflect their inability to cross the cell membrane. Absence of Al–$PcS_{3,6}$ in V-79 cells, even at elevated dye concentrations and prolonged incubation periods, was previously confirmed by fluorescence microscopy (Paquette *et al.*, 1988). *In vitro*, such polar dyes accumulate only on the cell surface where they may exert their phototoxic effect.

Table 1. Cell uptake and photodecomposition of Al–NcS and Al–PcS*

Dye	Molecules/ V-79 cell (± SD)	Percent photodecomposition (± SD)
Al–$NcS_{3,7}$	1.10 (±0.07) × 10^7	17.3 (±1.4)
Al–$NcS_{1,9}$	42.46 (±1.36) × 10^7	14.3 (±0.6)
Al–$PcS_{3,6}$	0.80 (±0.06) × 10^7	—
Al–$PcS_{2,0}$	13.08 (±0.80) × 10^7	—

*Chinese hamster V-79 cells were incubated with 70 μM of the Nc or Pc dye at 37°C for 1 h followed by a 8 min exposure to red light. Dye uptake by the cells was determined before and after irradiation.

In contrast, the more hydrophobic Al–$NcS_{1,9}$ fraction accumulated into V-79 cells 38 times better than the highly sulfonated Al–$NcS_{3,7}$ fraction and 3.2 times more efficiently than the equivalently sulfonated Al–PcS_2 fraction (Table 1). This high cellular accumulation likely reflects the distinct amphiphilic property of the assymetric mono- and disulfonated dyes. Accordingly, the lack of phototoxicity of the mainly disulfonated Al–$NcS_{1,9}$ fraction does not result from lack of cell penetrating properties of the dye.

Intracellular photostability

The same procedure was followed as described for the cell uptake studies except that the irradiation period was prolonged from 4 to 8 min (72 kJ/m^2). After extraction, the amount of dye was measured by its fluorescence, and recovered dye levels were compared to those from non irradiated cells (Table 1). The Al–$NcS_{3,7}$ and Al–$NcS_{1,9}$ fractions showed similar intracellular photostability, 17.3 and 14.3% dye decomposition upon cell irradiation, respectively. Since 2.7 times less red light fluence (27 kJ/m^2) was used during the photocytoxocity assay, this limited photodecomposition cannot account for the lack of photocytotoxicity exhibited by the Al–NcS preparations. Thus, the apparent photostability of the sulfonated naphthalocyanines most likely reflects extensive cellular aggregation of the dye, which in turn would explain the lack of a photocytotoxic response.

L-Tryptophan oxidation

Chemical quenching of $^1O_2$ by L-tryptophan followed by quantitation of the resulting hydroperoxide products (HPPI) is an indirect way to measure the capacity of the various dye preparations to generate $^1O_2$. Al–PcS_2 gave a Φ(HPPI) of 1.38 × 10^{-3} (Table 2), which is within the range of earlier Φ values reported for a series of Ga–PcS (Wagner *et al.*, 1987). The Al–$NcS_{3,7}$ and Al–$NcS_{1,9}$ fractions exhibited only slightly lower efficiencies to oxidize L-tryptophan with Φ(HPPI) of 0.89 × 10^{-3} and 0.85 × 10^{-3}, respectively. Furthermore, the 1.6 times lower efficiency for $^1O_2$ production of the Al–$NcS_{1,9}$ *vs* the Al–PcS_2, should, at the cellular level, largely be compensated for by the 3.2 times

Table 2. Quantum yield for the formation of L-tryptophan oxidation products (HPPI) after exposure of L-tryptophan to Al–NcS or Al–PcS and red light

Dye	Φ(HPPI) × 10^3
Al–$NcS_{3,7}$	0.89
Al–$NcS_{1,9}$	0.85
Al–$PcS_{2,0}$	1.38

higher cell uptake of the former. Thus, lack of phototoxicity of disulfonated Al–NcS towards V-79 cells cannot be explained by limited cell uptake, by intracellular stability, nor by lack of $^1O_2$ generating properties of this dye. These data suggest that the lower sulfonated Al–NcS are highly aggregated in the intracellular environment to yield photoinactive complexes or, alternatively, that these dyes are strongly associated with non-vital cellular components which readily quench any $^1O_2$ generated.

CONCLUSION

In these studies with V-79 Chinese hamster cells, we have evaluated phototoxicity, toxicity, cell uptake, intracellular stability, and the capability to generate $^1O_2$, of Al–NcS and Al–PcS sulfonated to different degrees. Whereas all underlying parameters leading to photosensitized cell killing are similar for both the naphthalo- and phthalocyanine dyes, the actual photocytoxicity of the two related classes of dyes was strikingly different. While both Al–PcS_2 or Al–NcS_2 were good generators of $^1O_2$, showed good cell uptake and sufficient intracellular photostability, only the Pc dye exhibited strong photocytotoxicity. Thus, lack of photoactivity of the Al–NcS_2 must involve another variable, such as close association with non-vital cell constituents or formation of photoinactive adducts and aggregates. The lack of *in vitro* activity does not exclude possible *in vivo* activities of the naphthalocyanine dyes since the latter does not necessarily require neoplasmic cell uptake. Furthermore, the lower photostability of the Nc dyes could limit unwanted secondary effects such as skin toxicity. Finally, the availability of inexpensive diode lasers, which deliver light at wavelengths compatible with the *Q* band absorption of naphthalocyanines, combined with the above considerations, warrants further *in vivo* studies on the potential of Nc dyes as photosensitizers for the PDT of cancer.

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