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

BENOIT PAQUETTE, HASRAT ALI, REJEAN LANGLOIS and JOHAN E. VAN LIER*
MRC Group in the Radiation Sciences, Faculty of Medicine, University of Sherbrooke,
Sherbrooke, Quebec, Canada J1H 5N4

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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 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 porphin analogs with red-shifted absorption maxima as compared to structural analogous phthalocyanines (Pc) (Fig. 1) are of particular interest, distinguishing them 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 O2, 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 photosensitizer 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–PcSx) 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, λmax, 674 nm, ε 19 x 10^4 M^-1 cm^-1; Al–NcS, λmax, 771 nm, ε 16 x 10^4 M^-1 cm^-1.

Mono- to tetrasulfonated aluminum naphthalocyanine (Al–NcS). Chloroaluminum naphthalocyanine (AlCl–Nc) was prepared by condensation of 2,3-dicyanophthalene and AlCl3 (anhydrous) at 220–280°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 H2SO4 containing 15% free SO3) 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)

*To whom correspondence should be addressed.

†Abbreviations: DMF, N,N-dimethylformamide; HPPt, 3a-hydroperoxy-1,2,3,3a,8,8a-hexahydropropyrole(2,3b) indole-2-carboxylic acid; Na, naphthalocyanine; NeSx, mono- to tetrasulfonaphthalocyanine; PBS, phosphate buffered saline; Pc, phthalocyanine; PcSx, 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_2 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.

WAVELENGTH IN nm

Absorbance IN O.D.

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 μl of oleum and 1.5 μl of 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 μl 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_4 fractions was determined by HPLC in an identical manner as described for the analogous Al-PcS_4 (Ali et al., 1988), except that the degree of sulfonation required), poured onto crushed ice and worked up in the usual manner.

Mixed Al-NcS (100 mg) in 100 μl 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_4 fractions was determined by HPLC in an identical manner as described for the analogous Al-PcS_4 (Ali et al., 1988), except that...
washed with 600 μM 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, MgCl₂ 10 mM, CaCl₂ 1 mM, pH 7.8). After three freeze-thaw cycles in liquid nitrogen, cell debris was incubated in the dark overnight with 0.5 A₅₅₀ unit of proteinase K (Sigma) and diluted in 100 μl of Tris buffer. DNA was digested for 2 h at 37°C with 1 A₅₅₀ 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/H₂O (95:5) was measured after 1 h stabilization period. AI-NcS and Al-PcS fluorescence was measured with a SLM-Aminco SPF-500C spectrophotofluorometer (AI-NcS: λₑ₅₄₀ = 760 nm, λₑ₇₆₀ = 790 nm; AI-PcS: λₑ₅₄₀ = 660 nm, λₑ₇₆₀ = 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 O₂ in homogenous aqueous solution yields a pair of characteristic hydroperoxides which have been characterized as the isomeric cis and trans 3α-hydroxy-1,2,3a,8,8α-hexahydropyrrolo[2,3b]indole-2-carboxylic acids (HPPI) (Langlois et al., 1986). HPPI is readily quantified by a colorimetric procedure using Fe²⁺ and xylenol orange. Fe²⁺ is oxidized to yield a Fe²⁺-xylenol orange complex with a λₑ₅₄₀ at 540 nm (Gupta, 1973). We have previously used this reaction to compare the O₂ generating capacity of differently sulfonated Ga-PcS and shown that the Φ(HPPI) is directly proportional to the Φ(O₂) (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 H₂SO₄, one part of 10⁻² M xylenol orange tetrasodium salt and nine parts of purified water (conductivity < 0.25 × 10⁻⁶ Ω⁻¹ cm⁻¹). L-Tryptophan (2.0 mM) and the Al-PcS or Al-NcS dye (3.5 μM) in MeOH/H₂O (95:5), saturated with O₂ by purging for 5 min, were irradiated for 1 h with red light. Prior to use, dye solutions were purified twice using Fe³⁺ and xylenol orange. Fe³⁺ is oxidized to yield a Fe³⁺-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–NcS₁₅ fraction, containing mainly tri- and tetrasulfonated dye, showed little photocytotoxicity with 73.7% cell mortality at 70 μM. 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 LD₅₀ of 38 μM was reported for Al–PcS₁₅ (Paquette et al., 1988) while Ga–PcS showed only slight phototoxicity, even at elevated concentrations of 200 μM (Brasseur et al., 1987).

Surprisingly, the hydrophobic Al–NcS₆½ fraction showed only 19% cell death at 100 μM, either with or without exposure to red light. This contrasts...
strikingly with our earlier observations on the analogous Al-PC$_{52}$, which induced 90% cell death at 1.7 \( \mu M \) (LD$_{50}$) after exposure to red light, without detectable dark toxicity (Paquette et al., 1988). Our failure to observe photocytotoxicity with the sulfonated 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 same 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-Nc$_{5,7}$ and Al-Pc$_{5,8}$, 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-Pc$_{5,8}$ 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

<table>
<thead>
<tr>
<th>Dye</th>
<th>Molecules/ V-79 cell (± SD)</th>
<th>Percent photodecomposition (± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Al-Nc$_{5,7}$</td>
<td>1.10 (±0.07) ( \times 10^7 )</td>
<td>17.3 (±1.4)</td>
</tr>
<tr>
<td>Al-Nc$_{5,9}$</td>
<td>42.46 (±1.36) ( \times 10^7 )</td>
<td>14.3 (±0.6)</td>
</tr>
<tr>
<td>Al-Pc$_{5,8}$</td>
<td>0.80 (±0.06) ( \times 10^7 )</td>
<td>—</td>
</tr>
<tr>
<td>Al-Pc$_{5,9}$</td>
<td>13.08 (±0.80) ( \times 10^7 )</td>
<td>—</td>
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</tbody>
</table>

*Chinese hamster V-79 cells were incubated with 70 \( \mu M \) 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-Nc$_{5,9}$ fraction accumulated into V-79 cells 38 times better than the highly sulfonated Al-Nc$_{5,7}$ fraction and 3.2 times more efficiently than the equivalently sulfonated Al-Pc$_{5,7}$ 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-Nc$_{5,9}$ 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-Nc$_{5,7}$ and Al-Nc$_{5,9}$ 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 photocytotoxicity 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 phototoxic response.

### 1-Tryptophan oxidation

Chemical quenching of $^1$O$_2$ 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 $^1$O$_2$. Al-Pc$_{5,7}$ gave a $\Phi_{(HPPI)}$ of \( 1.38 \times 10^{-3} \) (Table 2), which is within the range of earlier $\Phi$ values reported for a series of Ga-PcS (Wagner et al., 1987). The Al-Nc$_{5,7}$ and Al-Nc$_{5,9}$ fractions exhibited only slightly lower efficiencies to oxidize L-tryptophan with $\Phi_{(HPPI)}$ of 0.89 $\times 10^{-3}$ and 0.85 $\times 10^{-3}$, respectively. Furthermore, the 1.6 times lower efficiency for $^1$O$_2$ production of the Al-Nc$_{5,9}$ vs the Al-Pc$_{5,7}$ should, at the cellular level, largely be compensated for by the 3.2 times
higher cell uptake of the former. Thus, lack of photocytotoxicity of disulfonated Al-NcS towards V-79 cells cannot be explained by limited cell uptake, by intracellular stability, nor by lack of \( O_2 \) 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 photocytotoxicity, 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 naphthal- and phthalocyanine dyes, the actual photocytotoxicity of the two related classes of dyes was strikingly different. While both Al-PcS or Al-NcS 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 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 neoplastic 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.

**REFERENCES**


