Photodynamic properties of naphthosulfobenzoporphyrazines, novel asymmetric, amphiphilic phthalocyanine derivatives

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Abstract

Metallo naphthosulfobenzoporphyrazines sulfonated to different degrees (M-NSBP) were prepared, and their potential as photosensitizers for the photodynamic therapy (PDT) of cancer was evaluated. M-NSBP can be viewed as hybrid molecules between sulfophthalocyanines and naphthalocyanines resulting in distinct differences in the absorption spectra between the mono-through tetrasulfonated derivatives. This feature greatly facilited their purification. Using V-79 Chinese hamster cells *in vitro*, the disulfonated derivatives were found slightly more photoactive than the hydrophilic trisulfonated derivatives while the monosulfonated derivative was inactive, in spite of a sixfold higher cell uptake. In the case of the di- and trisulfonated derivatives, differences in phototoxicity correlated well with their relative cell uptake. Substitution of Al for Zn had little effect on the extent of phototoxicity of the M-NSBP. In vitro PDT of the EMT-6 cells after *in vivo* dye administration, revealed a similar potency for direct cell killing between the di-and trisulfonated AlOH–NSBP, while the monosulfonated analog was inactive. PDT with the amphiphilic disulfonated AlOH–NSBP on the EMT-6 mammary tumor in BALB/c mice induced a significant tumor response, while the monosulfonated derivative was much less active.

Keywords: Naphthosulfobenzoporphyrazine, photosensitizer, photodynamic therapy, V-79 Chinese hamster cells, EMT-6 murine mammary tumor cells, phthalocyanine derivative.

1. Introduction

Photodynamic therapy (PDT) of cancer, using a mixture of non-toxic hematoporphyrin derivatives as the photosensitizer and 630 nm red light, is currently in Phase III clinical trials (for recent reviews, 1–3). PDT could be rendered more efficient through the use of photosensitizers which are more selectively retained by neoplastic tissues while being more readily eliminated from non-target tissues such as the skin, and which absorb more strongly towards the red end of the visible spectrum. The

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rationale for the latter is that light above 680 nm allows for deeper penetration into biological tissues [4] as well as the availability of less expensive and more reliable light sources at these higher wavelengths.

Phthalocyanines (Pc) are promising dyes for PDT since they exhibit absorption bands above 650 nm. Within this family, the potential use of sulfonated phthalocyanines (PcS) has been well documented, particularly since their hydrosolubility makes them suitable for biological evaluation (for recent reviews see refs. 5–7). The metallo sulfophthalocyanines (M–PcS) are prepared either via direct sulfonation of the non-substituted macrocycle, or by condensation of phthalic/sulfophthalic acid mixtures. M–PcS sulfonated to different degrees are purified by medium-pressure reverse-phase column chromatography to yield fractions containing isomeric products with varying degrees of homogeneity. The degree of sulfonation of these fractions can be determined by reverse-phase high pressure liquid chromatography (HPLC) whereas oxidative degradation followed by HPLC analysis of the phthalamide/sulfophthalamide ratio can provide the average number of sulfonate groups per phthalocyanine molecule [8].

A novel class of phthalocyanine analogs, the metallo naphthosulfobenzoporphyrazines (M–NSBP), has recently been reported [9]. M–NSBP can be viewed as consisting both of PcS and non-substituted naphthalocyanine (Nc) moieties (Fig. 1). Because of the perturbation of the (na)phthalocyanine D_{4h} symmetry and the modification of the acennalation, the degree of sulfonation of the different condensation products can readily be deduced from the electronic spectra which shift from maxima around 680–720 nm, in the case of the trisulfonated M–NSB_3P, to major absorption bands between 720–770 nm, in the case of the monosulfonated M–N_3SBP [10]. Moreover, mixtures of isomeric M–NSBP sulfonated to the same degree are readily recognized by their identical absorption spectra, which greatly facilitates their purification. The presence of both sulfobenzo as well as naphtho groups on the same macrocycle enhances the amphiphilicity, a property which has been shown to improve cell penetration and photodynamic activity [11–14]. In this paper, we report on various biological properties of these dyes related to their potential use for the PDT of cancer.

2. Materials and methods

Sulfolane and naphthalene-2,3-dicarboxylic acid were purchased from Aldrich, aluminium chloride from Merck, 4-sulfophthalic acid from Kodak, urea, zinc diacetate and ammonium molybdate from Prolabo, France. HPLC grade solvents were obtained either from Fisher Scientific, or SDS, France. UV-visible absorption spectra were recorded on Varian 2000 and Perkin-Elmer Lambda 5 spectrophotometers. Fast atom bombardment (FAB) and plasma desorption mass spectra (PD-MS) were kindly provided by Dr. J. Favre-Bonvin (Service Central d'Analyse, CNRS, Lyon, France) and ion-spray mass spectra (IS-MS) by Dr. B. Shushan (SCIEX, division of MDS Health Group Ltd., Thornhill, Ontario, Canada). IS-MS were obtained on samples dissolved in 2 mM NH_4OAc in acetonitrile/water (1:1), infused at 5 μl min^{-1}. The HPLC thermospray mass spectra (TS-MS) were obtained with the HP 5988A quadrupole mass analyzer (Hewlett-Packard) and a Vestec (Houston, TX, USA) thermospray interface equipped with a CC 100 Cryocool immersion cooler, of our faculty.

2.1. Synthesis of M–NSBP

The procedure has been previously described for the Zn–NSBP series [9]. Briefly, the monosodium salt of 4-sulfophthalic acid (2.68 g, 10 mmol), naphthalene-2,3-

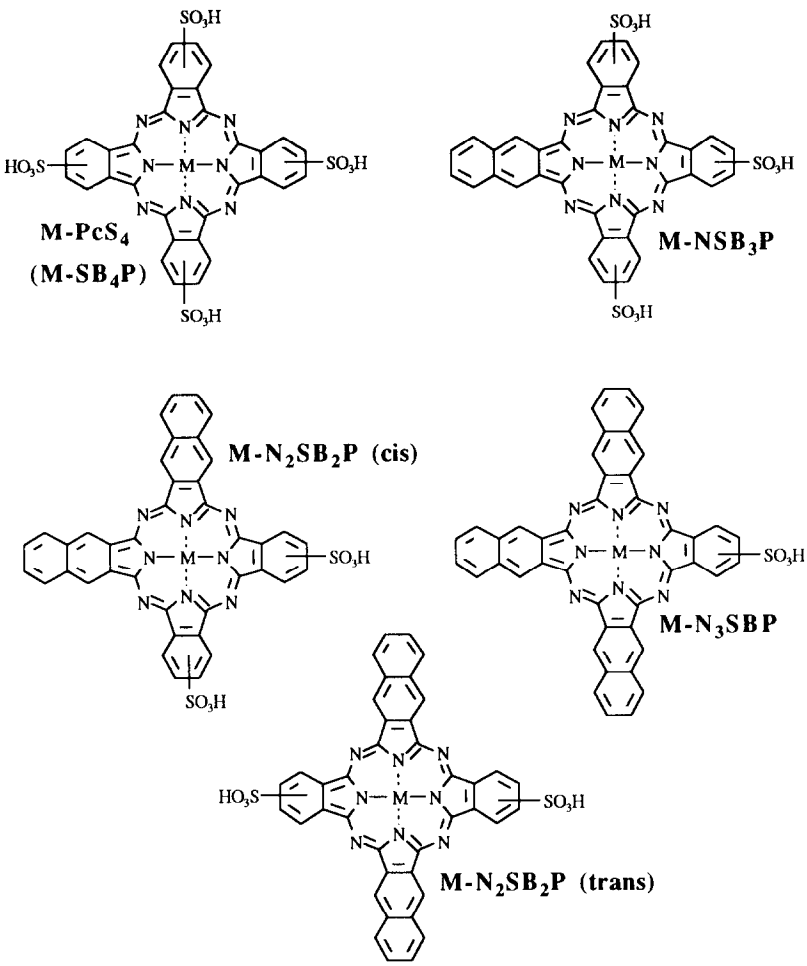

Fig. 1. Chemical structure of M–PcS_4 or M–SB_4P and M–NSBP: M = Zn or AlOH.

dicarboxylic acid (2.16 g, 10 mmol), urea (5.4 g, 90 mmol), zinc acetate (0.92 g, 5 mmol) or aluminium chloride (0.67 g, 5 mmol) and ammonium molybdate (4 mg, 0.02 mmol) were dissolved in 15 ml sulfolane (0.16 mol). The material was heated progressively from 140 °C to 270–290 °C over a period of 20 min on a Wood bath and kept at the final temperature for 6–7 h. The resulting sticky black cake was washed with acetone. Volatile impurities were removed by sublimation of the crude powder (2 g) at 250–260 °C under 0.1 mm Hg vacuum. The residue (1.5 g) was dispersed in two 50 ml plastic centrifuge tubes with 20 ml 10 mM sodium acetate, pH 5 containing 10% DMF (solvent A), following which 5 ml 1 M NaOH and 25 ml MeOH/DMF 9:1 (solvent B) were added. After sonication for 15 min, the solution was centrifugated (1500 g, 20 min) and the pellet dissolved in 50 ml of 100% solvent B. After another 15 min of sonication followed by centrifugation, the supernatant was collected and pooled with the first centrifugation supernatant, and neutralized with HCl pending medium pressure chromatography.

2.2. Preparative chromatography and analytical HPLC

M–NSBP were purified on a 30 cm long by 4 cm ID glass column packed with C-18 reverse-phase, particle size 25–40 μm (Macherey-Nagel) fitted with a 10 cm long by 2 cm ID precolumn. Elution was carried out by a stepwise gradient of 100% solvent A to 100% solvent B at a flow rate of 2–3 ml min^{-1}. A concentrated dye solution (200 mg) was loaded onto the column with a FMI model RP-SY pump. Eluting dyes were monitored via their visible electronic spectra and their analytical HPLC profiles. Metallo mononaphthotrisulfobenzoporphyrazines (M–NSB_3P) were desalted by re-chromatography on the same column in water (400 ml) and the trisulfonated dyes were eluted with 100% MeOH. The dinaphthodisulfobenzoporphyrazine (M–N_2SB_2P) and trinaphthomonosulfobenzoporphyrazine (M–N_3SBP) were dissolved in distilled water, precipitated at 4 °C (18 h), and washed with 1N HCl and distilled water. Relative yields for the AlOH–NSBP series were as follows: AlOH–PcS_4 13%; AlOH–NSB_3P, 31%; AlOH–N_2SB_2P, 33%; AlOH–N_3SBP, 23%; for the Zn–NSBP series: Zn–PcS_4, 13%; Zn–NSB_3P, 46%; Zn–N_2SB_2P, 29%; Zn–N_3SBP, 12%.

Analytical HPLC was conducted on a 25 cm long by 0.94 cm ID semi-preparative reverse-phase column packed with ODS-2 spherisorb, 5 μm (CSC, Montreal), operated at a flow rate of 2 ml min^{-1} with a linear gradient (25 min) of 0–95% MeOH in 10 mM sodium acetate buffer pH 5, followed by a 5 min isocratic step. Eluting dyes were detected by their absorption at 680 or 700 nm. In this manner we obtained homogeneous fractions consisting of isomers with the same degree of sulfonation, *i.e.* from the metallo tetrasulfophthalocyanine M–PcS_4 or M–SB_4P to the trinaphthomonosulfobenzoporphyrazine (M–N_3SBP).

2.3. Characterization of the dyes

The isomeric M–NSBP preparations are identified by their HPLC profiles and retention times (Fig. 2), identical and characteristic absorption maxima of the individual isomers in each preparation with the same degree of sulfonation (Fig. 3) and characteristic mass spectral ions. AlOH–PcS_4 (AlOH–SB_4P): HPLC $t_R < 10$ min; λ_{max} (DMF) 680 nm (ϵ 2×10^5); TS-MS (*m/z*) 876 (M^-), 918 ($M-H_2O$ + acetate)$^-$. AlOH–NSB_3P: HPLC t_R 12–18 min; λ_{max} (DMF) 353 nm (ϵ 6.2×10^4), 626 nm (ϵ 2.5×10^4), 692 nm (ϵ 9.1×10^4), 714 nm (ϵ 7.7×10^4); TS-MS (*m/z*) 846 (M^-), 888 ($M-H_2O$ + acetate)$^-$. AlOH–N_2SB_2P: HPLC t_R 19–24 min; λ_{max} (DMF) 351 nm (ϵ 7.3×10^4), 657 nm (ϵ 3.1×10^4), 728 nm (ϵ 9.9×10^4); IS-MS (*m/z*) 815.3 $(M - H)^-$; TS-MS (*m/z*) 816 (M^-), 858 ($M-H_2O$ + acetate)$^-$. AlOH–N_3SBP: HPLC t_R 27-30 min; λ_{max} (DMF) 347 nm (ϵ 6.9×10^4), 738 nm (ϵ 8.7×10^4), 762 nm (ϵ 7.4×10^4); FAB-MS (*m/z*) 770 $(M-OH)^+$. Zn–PcS_4 (Zn–SB_4P): HPLC t_R 10-15 min; λ_{max} 680 nm (ϵ 2×10^5). Zn–NSB_3P: HPLC t_R 16-20 min; λ_{max} (DMF) 347 nm (ϵ 5.5×10^4), 621 nm (ϵ 2.4×10^4), 687 nm (ϵ 8.7×10^4), 704 nm (ϵ 8.1×10^4); PD-MS (*m/z*) 913 $(M+2Na)^+$, 890 $(M+Na)^+$, 869 $(M+H)^+$, 868 $(M)^+$, 787 $(M-SO_3H)^+$, 707 $(M-2SO_3H)^+$, 626 $(M-3SO_3H)^+$. Zn–N_2SB_2P: HPLC t_R 21–26 min; λ_{max} (DMF) 344 nm (ϵ 5.8×10^4), 643 nm (ϵ 2.7×10^4), 715 nm (ϵ 10.2×10^4); FAB-MS (*m/z*) 837 $(M-H)^-$. Zn–N_3SBP: HPLC t_R 26–29 min; λ_{max} (DMF) 338 nm (ϵ 4.1×10^4), 654 nm (ϵ 1.7×10^4), 727 nm (ϵ 4.8×10^4), 746 nm (ϵ 4.2×10^4); FAB-MS (*m/z*) 831.2 $(M+Na)^+$.

2.4. In vitro photocytotoxicity assay

Cell survival of Chinese hamster lung fibroblasts (line V-79) was determined by a colony forming assay as previously described [12]. Plated cells were incubated with 1 ml of dye in medium containing 1% serum for 1 h in the dark at 37 °C in 5% CO_2. After removal of the dye solution and washing with PBS, cells were refed with

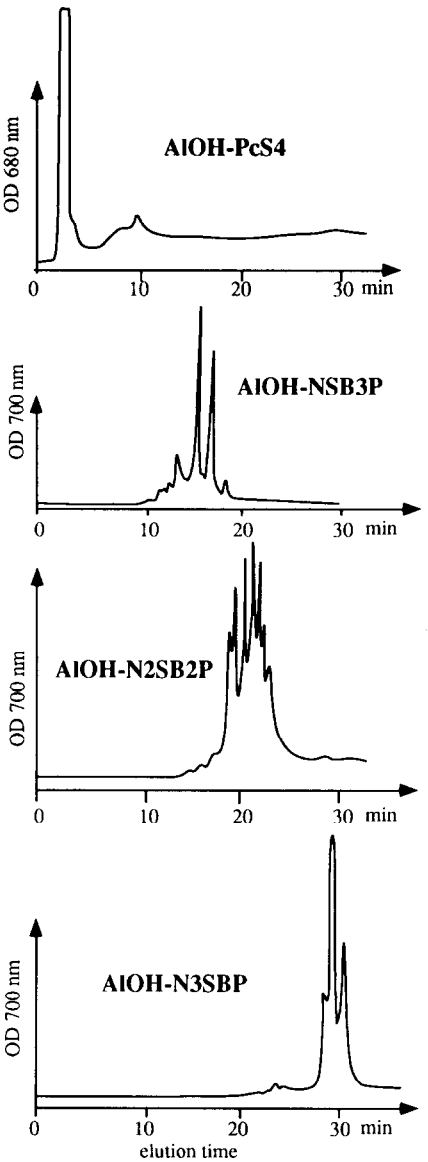

Fig. 2. Reverse-phase HPLC profiles of the mono- through tetrasulfonated AlOH–NSBP preparations. Individual isomers in each fraction are characterized by identical absorption spectra, a feature which facilitates the purification procedure.

growth medium and exposed for 4 min to red light from two 500 W tungsten/halogen lamps (Sylvania FCL) fitted with a refrigerated water filter and a red filter (26-4390, Ealing). The fluence over the absorption peaks of the photoactive monomeric dyes *i.e.* λ_{max} or mean of λ_{max} in the case of double Q bands, ± 30 nm, was 2.4 J cm^{-2}. The cells were then incubated at 37 °C in 5% CO_2 for 6–7 days. The dye concentration in μM required for 90% cell mortality, *i.e.* the extracellular LD_{90}, was used to quantify

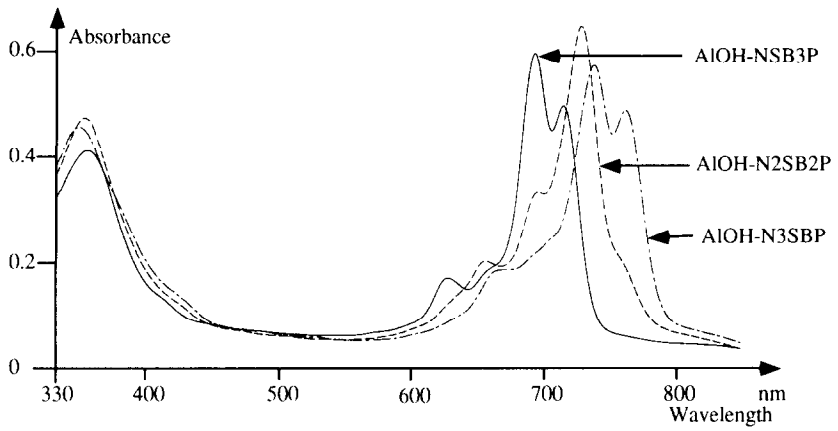

Fig. 3. Absorption spectra of AlOH–NSBP sulfonated to different degrees. The isomers in each preparation (Fig. 2) exhibit identical absorption spectra. Perturbation of the symmetry in the differently sulfonated dyes results in characteristic shifts of the absorption maxima.

the activity of a given dye preparation. Experiments were run at least three times with three dishes for each concentration point.

2.5. Cell uptake

About 3×10^6 V-79 cells were plated in 60 mm Petri dishes. After an incubation period of 3 h under 5% CO_2 to allow attachment, cells were incubated for 1 h with 10 μM of dye in 2 ml medium containing 1% serum, in the dark at 37 °C, under 5% CO_2. After removal of the dye solution and washing of the cells three times with PBS, cells were detached with a rubber policeman, collected by microcentrifugation in 1.5 ml Eppendorf tubes and resuspended in 1 ml 1 M NaOH. Cell suspensions were heated at 50 °C for 4 h and then diluted 40-fold in MeOH. The fluorescence was measured with a SLM-Aminco SPF spectrofluorometer (AlOH–NSB_3P: $\lambda_{ex}=684$ nm, $\lambda_{em}=717$ nm; AlOH–N_2SB_2P: $\lambda_{ex}=685$ nm, $\lambda_{em}=720$ nm; AlOH–N_3SBP: $\lambda_{ex}=730$ nm, $\lambda_{em}=760$ nm). Concentrations were calculated via standard curves. The extraction efficiency was evaluated by adding a known amount of dye in 1 ml 1 M NaOH to non-treated cells after the microcentrifugation step. All experiments were run in triplicate.

2.6. Ex vivo photocytotoxicity assay

Animal experiments were conducted in accordance with the recommendations of the Canadian Council on Animal Care. The model used to evaluate the tumoricidal potential of the various M–NSBP was the EMT-6 mouse mammary tumor implanted in the lower back of BALB/c mice. Monitoring of tumor growth, cell cycle and inoculation has previously been described [12]. The EMT-6 cell survival *in vitro*, after *in vivo* dye administration and tumor excision, was determined according to a protocol established by Henderson *et al.* [15]. Photosensitizers were solubilized in an emulsion of 10% Cremophor EL (Sigma), 3% propanediol, 0.9% NaCl saline and injected at a dose of 10 μmol kg^{-1} body weight ($\pm$200 μl) via the caudal vein of BALB/c mice bearing the EMT-6 tumor. After 24 h the mice were anesthetized with ether and killed. The tumors were washed with 70% ethanol, removed with sterile instruments and transferred to Petri dishes containing 5 ml PBS enriched with 1% penicillin (10.000 U ml^{-1}) - streptomycin (10 mg ml^{-1}) (Gibco). The tumors were minced and enzymatically

disaggregated at 37 °C for half an hour by stirring in the following mixture: $CaCl_2$ 10mM, proteinase K (Sigma) 6.5 U, micrococcal nuclease (Sigma) 3 U, collagenase (Sigma) 17 U, in 10 ml Hank's buffered saline solution. The digested preparation was then filtered through a 200 mesh sieve and centrifuged at 600 g for 5 min. Two hundred cells were placed in 6 cm Petri dishes and incubated for 3 h at 37 °C in 5% CO_2 in Waymouth's culture medium to allow adhesion to the plastic support. Cells were then illuminated as described under the *in vitro* section with a fluence from 2.5 to 50 J cm^{-2}. After illumination, cells were incubated for 6–7 days. The colonies were fixed with methanol, stained with crystal violet and counted. Plating efficiency was determined from the cell survival of five non-illuminated Petri dishes. From this value, the percentage of cell survival was calculated. *Ex vivo* experiments were independently repeated at least twice, with three dishes per point.

2.7. In vivo photocytotoxicity assay

Dye administration was done in the same manner as described above. After 24 h, tumors were exposed to 400 J cm^{-2} of red light (700–800 nm) from a 1000 W Xenon lamp, equipped with a 10 cm water filter, and LL-700 and LS-800 filters (Corion) cooled by air. The light was focused into a 8 mm diameter beam by optical lenses. The mean fluence rate was 240 mW cm^{-2}. Under these conditions no hyperthermia occurred during illumination [16]. Necrosis within 72 h after PDT, followed by tumor regression during the next two weeks, was recorded as a positive response. Six mice per dye dose were used.

3. Results

3.1. In vitro phototoxicity

Control V-79 cells exposed to red light (2.4 J cm^{-2}) without prior incubation with dye showed no significant mortality. Their plating efficiency was taken as the 100% cell survival. Cells incubated either with AlOH–NSBP up to 20 μM, or Zn–NSBP up to 25 μM, for 1 h without subsequent light exposure displayed no effects.

3.1.1. Effect of the degree of sulfonation

The effect of M–NSBP on V-79 cell survival after 1 h incubation with the dye followed by an exposure to red light (2.4 J cm^{-2}) are presented in Fig. 4 and the LD_{90} are summarized in Table 1. The disulfonated M–N_2SB_2P were the most efficient photosensitizers for cell killing with LD_{90} from 3.75–4 μM for Zn–N_2SB_2P and AlOH–N_2SB_2P, respectively. The more hydrophilic trisulfonated M–NSB_3P were slightly less photoactive with a LD_{90} of 6.3 μM for AlOH–NSB_3P and 5.75 μM for Zn–NSB_3P. In contrast, the extremely hydrophobic M–N_3SBP exhibited no photocytoxicity, even at 20 μM. These data are compared to literature values of M–PcS and M–NcS photoactivities obtained under the same conditions [11–13, 17] (Table 1).

3.1.2. Effect of the nature of the central metal

The nature of the central metal, *i.e.* Al or Zn, has very little effect on the *in vitro* phototoxicity of the M–NSBP. The Zn complexes are slightly more photoactive than the Al complexes and these differences are far less pronounced than those observed in the M–PcS series (Table 1).

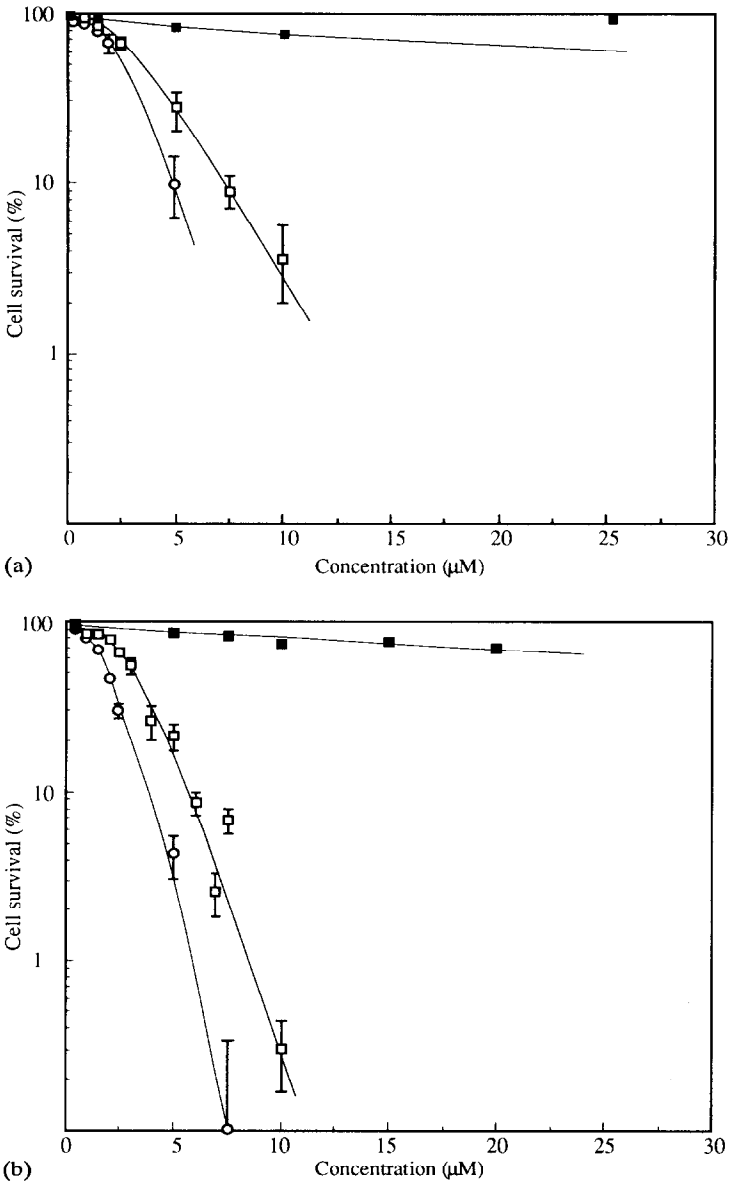

Fig. 4. Survival of V-79 cells after a 1 h incubation period with M–NSB_3P (□), M–N_2SB_2P (○), or M–N_3SBP (■) followed by exposure to red light. Data are the mean of three to five independent experiments run in triplicate. The vertical bars represent the standard error of the mean. (a) M = Zn; (b) M = AlOH.

3.1.3. Cell uptake

Uptake of AlOH–NSBP by V-79 cells is presented in Table 2. In order to correlate these data to photocytotoxicities, the incubation times were kept equal in both assays. Increased hydrophobicity resulted in increased cell uptake: the monosulfonated

TABLE 1

LD_{90} of M–NcS and M–NSBP for V-79 cell killing

Photosensitizer[a]	LD_{90} (μM)	Photosensitizer	LD_{90} (μM)
Zn–PcS_4[b]	60	AlOH–$PcS_{3.6}$[c]	38.3
Zn–NSB_3P	5.75	AlOH–NSB_3P	6.3
Zn–N_2SB_2P	3.75	AlOH–N_2SB_2P	4
Zn–N_3SBP	> 25	AlOH–N_3SBP	> 20
Zn–PcS_2[b]	0.2	AlOH–PcS_2[c]	1.7
Photofrin II[tm b]	1.7		

[a]Cells were incubated for 1 h with the dyes at various concentrations, rinsed and exposed to 2.4 J cm^{-2} red light.
[b]Data taken from [12].
[c]Data taken from [13].

TABLE 2

V-79 cell uptake of AlOH–NSBP after 1 h incubation at 10 μM

Photosensitizer	Cellular concentration 10^6 moles of dye/cell (SE)
AlOH–NSB_3P	48.2 (3.9)
AlOH–N_2SB_2P	73.3 (8.6)
AlOH–N_3SBP	327 (6.5)

SE = standard error.

AlOH–N_3SBP accumulated almost seven times more efficiently in V-79 cells than the corresponding trisulfonated AlOH–NSB_3P. It should be noted that the ratio between the LD_{90} of AlOH–NSB_3P and AlOH–N_2SB_2P parallels the relative cell uptake.

3.2. In vivo studies

3.2.1. Ex vivo phototoxicity

The potential for direct tumor cell killing during *in vivo* PDT with various AlOH–NSBP was investigated via an *in vivo/ in vitro* (*ex vivo*) protocol. In this assay, BALB/c mice bearing the EMT-6 tumor are intravenously injected with the photosensitizer, and this is followed by *in vitro* illumination of the isolated tumor cells. Mice which received 10 μmol kg^{-1} body weight (± 8 mg kg^{-1}) for the various dyes without subsequent exposure to red light did not show any apparent toxic reaction. Cell survival curves as a function of the red light fluence are presented in Fig. 5. The relative phototoxicities of the dyes in this *ex vivo* assay are similar to those observed under the *in vitro* conditions with the V-79 cells. Thus, at 50 J cm^{-2}, AlOH–N_3SBP induces only 50% cell death, while the AlOH–NSB_3P and AlOH–N_2SB_2P exhibit LD_{90} at 12 J cm^{-2} and 13.7 J cm^{-2} respectively.

3.2.2. In vivo phototoxicity

No tumor necrosis was observed in control animals treated with dye alone, or in animals exposed to light without prior injection of dye. Tumor response pattern to photosensitization with the M–NSBP were similar to those described for various M–PcS

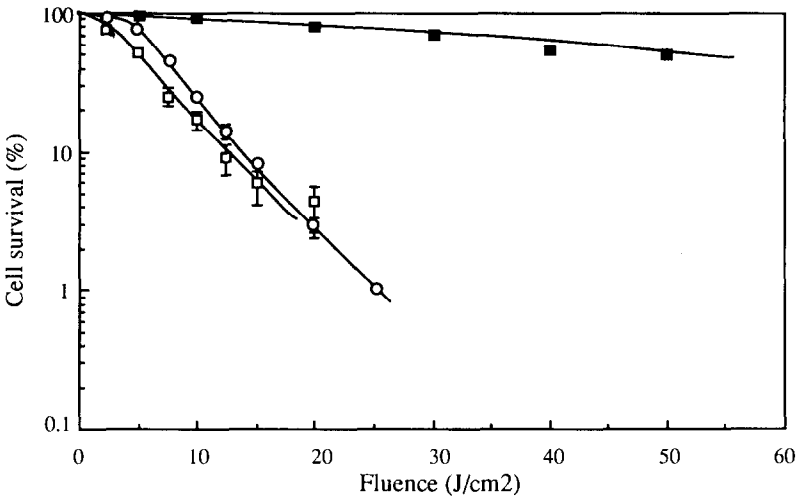

Fig. 5. *Ex vivo* EMT-6 tumor cell survival as a function of the red light fluence. Cells were isolated from tumors excised from BALB/c mice which were injected 24 h earlier with 10 μmol kg^{-1} of AlOH–NSB_3P (□), AlOH–N_2SB_2P (○), or AlOH–N_3SBP (■). Data are the mean of two or three independent experiments run in triplicate. The incertitude bars represent the standard error of the mean.

[16], *i.e.* extensive tumor necrosis within 72 h after PDT, followed by tumor regression. Tumor response induced by PDT with AlOH–N_2SB_2P and AlOH–N_3SBP at 1 μmol kg^{-1} body weight (0.8 mg kg^{-1}) was 83% and 33% ($n=6$) respectively. Under similar conditions (400 J cm^{-2}, 600–650 nm), Photofrin II^{tm} gave 33% tumor response at 5 mg kg^{-1} [18].

4. Discussion

M–NSBP (Fig. 1) can be considered as structural intermediates between M–PcS and M–Nc and as such their photoactivities add complementary data to our understanding of possible PcS structure–activity relationships. In contrast to the corresponding M–PcS, the central metal ion in M–NSBP affects their phototoxicity only slightly. This is surprising, since the Zn(II) ion confers a planar structure to the M–NSBP, which favors aggregation, while the Al(III) ion, with an out of plane, covalently bonded, axial coordinate, renders the molecule less prone to stacking. Thus, the lack of a pronounced biological effect associated with metal substitution suggests that the three-dimensional structure of the M–NSBP is not important for their photocytotoxicity.

The degree of sulfonation of M–NSBP strongly affects their biological activities and in this series, the isomeric disulfonated M–N_2SB_2P are the most photocytotoxic towards V-79 cells. This parallels previous observations on M–PcS [5], and sulfonated derivatives of tetraphenylporphine [19]. In the case of the M–PcS it has been shown that the degree of sulfonation does not affect the photochemical properties of the monomeric form of the photosensitizer [20]. However, sulfonation modifies the tendency of the M–PcS to aggregate, and since only the monomeric dyes are capable of the photogeneration of singlet oxygen [21], the degree of sulfonation strongly affects the photocytotoxicity indirectly [5]. Apart from the tendency to aggregate, sulfonation

mainly affects solubility and the capacity of the dyes to penetrate the cell membrane. Indeed, the disulfonated phthalocyanines exhibited the best photoactivity towards V-79 cells (Table 1) and this has been attributed to the amphiphilic nature of these dyes [13]. Differences in activities between the amphiphilic AlOH–N_2SB_2P and the hydrophilic AlOH–NSB_3P correlate well with cell uptake (Tables 1 and 2) which, combined with similarities in their singlet oxygen quantum yields in organic solvents [10], suggests that both dyes have identical action mechanisms and targets in the cell.

The monosulfonated Zn– and AlOH–N_3SBP lack photocytotoxicity, even at elevated concentrations of 20 μM. In the Zn–PcS series, the monosulfonated dye also is less active than the disulfonated analog but still exhibits substantial photocytotoxicity [12]. The lack of photocytotoxicity of the AlOH–N_3SBP cannot be explained by limited cell uptake, V-79 cells accumulate this dye 4.5 times better than the highly photocytotoxic AlOH–N_2SB_2P. Compared to monosulfonated M–PcS, the hydrophobicity of the AlOH–N_3SBP is increased due to the presence of the bulky, hydrophobic, naphtho constituents which may increase the tendency to aggregate and thus explain the loss of phototoxicity. These findings are in agreement with recent observations that, under *in vitro* conditions, M–NcS are substantially less phototoxic than the corresponding M–PcS [17, 22, 23].

The mechanism of tumor inactivation during and after PDT with phthalocyanine derivatives remains controversial. It is now well established that in the case of the hematoporphyrin derivatives vascular damage is the dominant factor in tumor destruction [24–26]. *Ex vivo* experiments provide information on the potential for direct photosensitizing effects on neoplastic cells during PDT, in the absence of the usual oxygen and light limitations of the *in vivo* environment. Henderson *et al.* [27] showed that a low *ex vivo* LD_{90} of a photosensitizer, *i.e.* a high potential for direct tumor cell killing, correlates to the requirement for a high light dose to induce vascular shutdown. Photofrin II^{tm} displayed little *ex vivo* activity which confirms vascular damage as the main cause of tumor necrosis after PDT. The AlOH–N_3SBP lacked phototoxicity in the *ex vivo* model, but both the AlOH–NSB_3P and the AlOH–N_2SB_2P showed a good potential for direct tumor cell killing, with similar *ex vivo* LD_{90} values. Thus, tumor regression after PDT with the two latter dyes could involve to a larger extent direct neoplastic cell killing as compared to the same tumor response observed after PDT with Photofrin II^{tm}.

5. Conclusion

NSBP can be viewed as hybrid molecules of PcS and Nc. As a result of the characteristic spectral fingerprints of the individual components, the mono- through tetrasulfonated products are readily fractionated by chromatographic procedures. The amphiphilic character of the M–NSBP is enhanced as compared to the corresponding PcS due to the added naphtho groups; however the latter substituents also augment the tendency to aggregate. This is reflected in the lack of photodynamic activity of the monosulfonated M–N_3SBP and the good photosensitizing activities of the di- and trisulfonated analogs.

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