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Photodynamic Properties of Amphiphilic Derivatives of Aluminum Tetrasulfophthalocyanine¶

Cynthia M. Allen, Réjean Langlois, Wesley M. Sharman, Carole La Madeleine and Johan E. Van Lier*

CIHR Group in the Radiation Sciences, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, Québec, Canada

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ABSTRACT

Photodynamic therapy (PDT) is a promising treatment modality that has recently been accepted in clinics as a curative or palliative therapy for cancer and other nonmalignant conditions. Phthalocyanines (Pc) are attractive photosensitizers for PDT because of their enhanced photophysical and photochemical properties. The overall charge and solubility of Pc play a major role in their potential usefulness for PDT. A series of amphiphilic derivatives of tetrasulfonated aluminum Pc ($AlPcS_4$) was prepared by substituting one of the four sulfonate groups with aliphatic side chains of 4, 8, 12 and 16 carbon atoms. The photodynamic properties of the derivatives were compared with those of $AlPcS_4$ and the adjacent disulfonated aluminum Pc. Parameters studied included reversed-phase high-performance liquid chromatography (HPLC) retention times, capacity to generate singlet oxygen ($^1O_2$), *in vitro* cell uptake and phototoxicity, as well as PDT response of transplantable EMT-6 tumors in mice. The monomerized $AlPcS_4$ derivatives showed similar or higher capacities to generate $^1O_2$ as compared with the parent $AlPcS_4$ as measured from relative L-tryptophan photooxidation yields. A549 cell uptake of the $AlPcS_4$ derivatives decreased in the following order: $AlPcS_4(C16) > AlPcS_4(C12) > AlPcS_4(C8) > AlPcS_4(C4)$. Human low-density lipoprotein at high concentrations (40 μg/mL) completely prevented uptake, whereas at 4 μg/mL uptake was decreased for the more lipophilic compounds and yet remained unaffected for the more hydrophilic dyes. Using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, A549 cell survival was assessed; it showed that photocytotoxic activity varied directly with the HPLC retention times, *i.e.* more hydrophilic compounds were less phototoxic. As $^1O_2$ yields were similar for the four substituted $AlPcS_4$ derivatives, it was postulated that the increased cytotoxic activity was caused by enhanced subcellular localization as a result of the long aliphatic side chains. These amphiphilic compounds proved to be photodynamically potent against the EMT-6 mouse mammary tumor model implanted in Balb/c mice. At dye doses of 0.2 μmol/kg and a fluence of 400 J/cm^2 complete tumor regression was observed with no morbidity. The substitution of $AlPcS_4$ with long aliphatic chains on the macrocycle greatly enhances its photodynamic efficacy both *in vitro* and *in vivo.*

INTRODUCTION

Ever since the observation of the lethal effects of acridine dyes on paramecia (1), interest in the use of light as a therapeutic tool has grown (2). The field of photodynamic therapy (PDT) has evolved from its early stages into a useful clinical tool in the treatment of both malignant and nonmalignant conditions. PDT involves the illumination of a photosensitizer with visible light, leading to the production of cytotoxic species such as reactive oxygen species that ultimately induce oxidative biological damage and cell death (3). First generation photosensitizers such as Photofrin® have gained clinical acceptance as therapeutic agents for a number of conditions that are easily accessible to light (4). However, such photosensitizers have important drawbacks including prolonged cutaneous retention and important skin photosensitivity (5,6). This invoked the search for new photosensitizers with improved chemical and biological properties. Among these are verteporfin, which has recently become an accepted treatment for age-related macular degeneration under the trade name Visudyne™, and aminolevulinic acid, a prodrug for the endogenous production of protoporphyrin IX as an accepted treatment for actinic keratoses (4,7).

Phthalocyanines (Pc) have been extensively examined as photosensitizers for PDT (7,8). These azoporphyrin derivatives have stronger absorbances at longer wavelengths than do porphyrins and tend to have improved photophysical and photochemical properties. Their chemical synthesis and structure permit the addition of substituents to both the central metal ion and the periphery of the hydrophobic Pc macrocycle, altering the physical properties of these photosen-

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*To whom correspondence should be addressed at: Department of Nuclear Medicine and Radiobiology, Faculty of Medicine, Université de Sherbrooke, Sherbrooke, QC J1H 5N4, Canada. Fax: 819-564-5442; e-mail: jvanlier@courrier.usherb.ca

Abbreviations: $AlPcS_{2adj}$, aluminum disulfophthalocyanine; $AlPcS_4$, aluminum tetrasulfophthalocyanine; CRM, Cremophor EL; EDTA, ethylenediamine-tetraacetic acid; FCS, fetal calf serum; HPLC, high-performance liquid chromatography; HPPI, 3α-hydroperoxy-1,2,3,3α,8,8α-hexahydropyrrolo[2,3β]indole-2-carboxylic acid; LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; $^1O_2$, singlet oxygen; PBS, phosphate-buffered saline; Pc, phthalocyanine; PDT, photodynamic therapy; TFA, trifluoroacetic acid.

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sitizers. Factors such as the structure, charge and hydrophobicity of a photosensitizer determine its interactions with itself and its biological surroundings, which in turn will determine its cellular uptake, subcellular localization and photocytotoxicity (9). It has been shown for instance that amphiphilic photosensitizers are generally more photodynamically active than are hydrophobic or hydrophilic molecules (10,11). Amphiphilicity describes photosensitizers having both hydrophobic and hydrophilic characteristics in different segments within the same molecule. Such structural features permit these distinct portions to interact differently with their biological environment while bestowing enhanced solubility, modulating aggregation and improving cellular uptake and intracellular targeting. It has been suggested that the favorable pharmacokinetic behavior needed to ensure tumor selectivity and rapid systemic clearance is related to the amphiphilic properties of the photosensitizer (2). A series of sulfonated tetraphenylporphines and sulfonated Pc display similar trends in photocytotoxicity with the more amphiphilic adjacent disulfonated photosensitizers exhibiting the highest toxicity (11–14). Interestingly, whereas the opposite and adjacent disulfonated tetraphenylporphines display identical hydrophobicity as measured by the partition coefficient, each features very different tumor localizing and photosensitizing properties because of varying amphiphilicity (12). Furthermore, despite having the highest levels of tumor cell uptake and the more important tumor selectivity, the tetrasulfonated tetraphenylporphine remains the least photodynamically active member of this family (15). This would seem to indicate the importance of amphiphilicity in the subcellular targeting of photosensitizers. Meanwhile, addition of *t*-butyl groups to trisulfonated Pc results in increased viral inactivation in red blood cell concentrates, indicating amphiphilicity enhances the photodynamic activity and targeting of these dyes (16). It has also been proposed that the more active fractions of Photofrin® may be the more amphiphilic ones (9). Hydrophobic photosensitizers tend to aggregate in solution (5,17,18), which decreases their photodynamic activity (17), whereas hydrophilic photosensitizers display decreased cell uptakes (11), target less photosensitive subcellular targets (15,19) and are rapidly cleared *in vivo* (20). Amphiphilic molecules, on the other hand, display more ideal physical and pharmacokinetic properties, which increases their utility as photosensitizers for PDT. This has initiated the synthesis of new amphiphilic photosensitizers along with the chemical modification of existing structures in order to modify their pharmacology, generally by altering their amphiphilic characteristics.

In the present study, a tetrasulfonated Pc was substituted with alkyl chains of varying lengths *via* a sulfonamide bond in order to modify the amphiphilicity of the molecule (Fig. 1). The cellular uptake and photocytotoxicity of the compounds obtained were determined and compared with various structural features of the photosensitizers including the hydrophobicity as determined by high-performance liquid chromatography (HPLC) retention times. Photodynamic treatment of EMT-6 tumors in Balb/c mice was also evaluated.

MATERIALS AND METHODS

Preparation of aluminum tetrakis(2-sulfonylchloro)phthalocyanine. Tetrasulfonated aluminum Pc ($AlPcS_4$) was synthesized *via* the condensation of 4-sulfophthalic acid monosodium salt in the presence of aluminum chloride and urea as described in the literature (21,22). $AlPcS_4$ (1 g, 1.12 mmol) was stirred in chlorosulfonic acid (4 mL) at 120°C for 3 h. The solution was cooled to 80°C, and thionyl chloride (6 mL, 82.6 mmol) was added and the reaction stirred at 80°C for another 2 h. After cooling to room temperature, the reaction mixture was poured on ice, and the desired AlPc tetrasulfonyl chloride was collected by centrifugation and filtration and was dried over P_20_5, giving 0.97 g of the product (90% yield) (23,24).

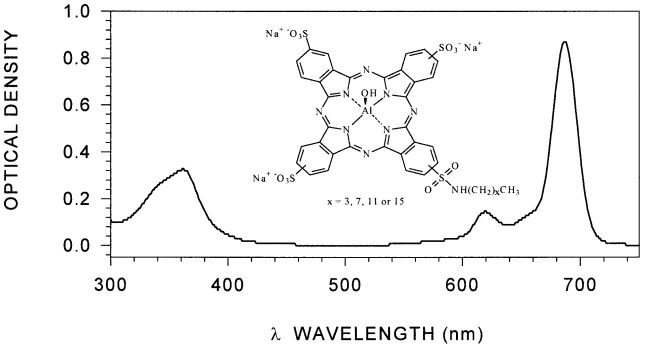

Figure 1. Pc chemical structure (inset). Absorption spectrum of $AlPcS_4$(C12) monomerized in DMF (100× dilution) encompassing both the Soret band (340 nm) and the Q band (680 nm).

Aluminum 2-butylaminosulfonyl-9(10),16(17),23(24)-trisulfophthalocyanine (C4). AlPc tetrasulfonyl chloride (0.2 g, 0.2 mmol) was added in small portions to a stirred solution of *n*-butylamine (73 mg, 1 mmol) in water (20 mL) and 0.1 *N* $NaHCO_3$ (10 mL, 1 mmol). The reaction was stirred for 16 h at room temperature. The pH of the solution was then adjusted to 10–11, and the solution was extracted with diethyl ether in order to remove unreacted *n*-butylamine. The aqueous layer was readjusted to pH 6–7, and the solvent was removed under reduced pressure. Purification was accomplished using a semipreparative chromatographic system composed of a glass fritted filter (60 mL, 3.0 cm diameter) filled with Polygosil 60-2540 C18 reversed-phase packing (Macherey-Nagel, Düren, Germany). The $AlPcS_4$–butylamine reaction was introduced on the semipreparative column using phosphate buffer pH 5.0 and was washed with 100 mL of this buffer solution. The desired product was then eluted with 100 mL of phosphate buffer–MeOH (8:2 vol/vol). The column was then washed with 100% MeOH to reactivate the C18 reverse phase packing. The purification was repeated using water and methanol for elution in order to remove the phosphate salts. Fractions were analyzed by HPLC with the different constitutional isomers for the C4, giving retention times in the range of 12.5–16.5 min (Table 1). HPLC analyses were conducted with a Shimadzu instrument (Kyoto, Japan) composed of an LC-600 pump, SP-6AV UV-visible detector, DGV-4A degasser, LPM-600 low pressure mixing system, a rheodyne injector and an EZChrom chromatography data system. The HPLC system consisted of a Radial-Pak cartridge (10 × 0.8 cm) filled with 4 μm Nova Pak C18 reversed-phase packing (Waters Corp., Milford, MA). The column was eluted at 1.5 mL/min with a linear gradient from 0 to 100% MeOH in 10 m*M* phosphate buffer, pH 5.0, over 30 min. The UV–visible detector was set at 680 nm.

The average number of sulfonate groups per Pc molecule was determined by an oxidative degradation procedure, followed by HPLC analysis of the various phthalimide fragments (26). In brief, 1 mg of the sulfonated Pc was dissolved in a minimal volume of water, and a few crystals of ammonium cerium sulfate were added. The solution was heated at 50–80°C until the characteristic green–blue color of the Pc disappeared (5–10 min). The colorless solution was then analyzed by HPLC. In this case, the system consisted of two Radial-Pak cartridges (10 × 0.8 cm) filled with 4 μm Nova Pak C18 reversed-phase packing. Elution was carried out at 1.5 mL/min with 0.1% trifluoroacetic acid (TFA) in water for 5 min, followed by a linear gradient from 0% to 100% methanol (0.02% TFA) over 30 min. The UV–visible detector was set at 215 nm. The 4-sulfophthalimide eluted at 13.2 min, whereas the 4-(butylaminosulfonyl)phthalimide eluted at 30.4 min. The peak area of the 4-sulfophthalimide was compared with that of the 4-(butylaminosulfonyl)

Table 1. Relative hydroperoxide (HPPI) yields as a result of L-tryptophan photooxidation in comparison with chromatographic mobilities on a C18 HPLC column

Compound	Retention time, R_t* (min)	Hydroperoxide formation ($AlPcS_4$ = 1)†		
		PBS	50% MeOH	1% CRM
$AlPcS_4$	8	1	1	1
$AlPcS_{2adj}$	24	0.335	0.761	1.36
C4	14	0.565	0.719	1.11
C8	20	0.311	0.838	1.17
C12	24	0.296	0.802	1.14
C16	29	0.135	0.819	n.a.‡

*The retention times on a reversed-phase C18 HPLC column (30 min linear gradient from 100% 10 m*M* phosphate buffer (pH 5) to 100% methanol at 1.5mL/min). Values represent the weighted average R_t of each fraction of isomeric products of the same structural composition.

†HPPI formation as a result of L-troptophan photooxidation when the AlPcS derivatives were solubilized in PBS, 50% MeOH and 1% CRM. The absolute values for the percent tryptophan oxidation after a 2 min irradiation of $AlPcS_4$ were 12.5, 20.0 and 6.7% in PBS, 50% MeOH and 1% CRM, respectively. The $^1O_2$ quantum yield for $AlPcS_4$ in phosphate buffer (1% Triton X) is reported to be 0.43 (25).

‡n.a., data not available.

phthalimide, giving a ratio of 75.7 to 24.3, confirming the desired monosubstitution of the $AlPcS_4$.

Aluminum 2-octylaminosulfonyl-9(10),16(17),23(24)-trisulfophthalocyanine (C8). A reaction procedure similar to that for the C4 was used. In this case, *n*-octylamine (5 equiv.) was used with acetone (20 mL) and 0.1 *N* $NaHCO_3$ (10 mL) acting as the reaction solvent. The reaction mixture was introduced onto the semipreparative column using phosphate buffer–MeOH (8:2 vol/vol), and the desired product was eluted using 100 mL of phosphate buffer–MeOH (6:4 vol/vol). HPLC analysis gave retention times for the four different constitutional isomers in the range of 17.5–22.0 min (Table 1). The average number of sulfonate groups per Pc molecule was determined as in the case of the C4 with the 4-(octylaminosulfonyl)phthalimide eluting at 36.9 min. The ratio of 4-sulfophthalimide to 4-(octylaminosulfonyl)phthalimide was 81.9/18.1, confirming the monosubstitution of the $AlPcS_4$.

Aluminum 2-dodecylaminosulfonyl-9(10),16(17),23(24)-trisulfophthalocyanine (C12). A reaction procedure similar to that used for the C4 was used. In this case, *n*-dodecylamine (5 equiv.) was used with acetone (80 mL) and 0.1 *N* $NaHCO_3$ (10 mL) acting as the reaction solvent. The reaction mixture was introduced onto the semipreparative column using phosphate buffer–MeOH (6:4 vol/vol), and the desired product was eluted using 100 mL of phosphate buffer–MeOH (4:6 vol/vol). HPLC analysis gave retention times for the four different constitutional isomers in the range of 21.5–26.0 min (Table 1). The average number of sulfonate groups per Pc molecule was determined as in the case of the C4 with the 4-(dodecylaminosulfonyl)phthalimide eluting at 40.5 min. The ratio of 4-sulfophthalimide to 4-(dodecylaminosulfonyl)phthalimide was 75.9/24.1, confirming the monosubstitution of the $AlPcS_4$.

Aluminum 2-hexadecylaminosulfonyl-9(10),16(17),23(24)-trisulfophthalocyanine (C16). A reaction procedure similar to that used for the C4 was used. In this case, *n*-hexadecylamine (5 equiv.) was used with acetone (160 mL), tetrahydrofuran (160 mL) and 0.1 *N* $NaHCO_3$ (10 mL) acting as the reaction solvent. The reaction mixture was introduced onto the semipreparative column using phosphate buffer–MeOH (6:4 vol/vol), and the desired product was eluted using 100 mL of phosphate buffer–MeOH (2:8 vol/vol). HPLC analysis gave retention times for the four different constitutional isomers in the range of 27.0–31.5 min (Table 1). The average number of sulfonate groups per Pc molecule was determined as in the case of the C4 with the 4-(hexadecylaminosulfonyl)phthalimide eluting at 42.4 min. The ratio of 4-sulfophthalimide to 4-(hexadecylaminosulfonyl)phthalimide was 83.0/17.0, confirming the monosubstitution of the $AlPcS_4$.

The photosensitizers were solubilized in phosphate-buffered saline (PBS) for both *in vitro* and *in vivo* experiments. Dissolving of (C16) was aided with trace amounts of MeOH. Previously, photosensitizer concentration has been determined using a molar extinction coefficient corresponding to the Pc Q band absorption peak. However in the present study, concentrations were determined in PBS using a molar extinction coefficient (ϵ) of 80 000 M^{-1} cm^{-1} at 340 nm. The Soret band molar extinction coefficient is independent of solvent and therefore unaffected by aggregation and remains unchanged within experimental error (5).

Aluminum 2, 9(10)-disulfonated Pc. The disodium salt of disulfonated aluminum Pc ($AlPcS_2$) featuring a hydroxyl axial ligand and enriched in isomers bearing sulfonate groups on adjacent benzo rings was synthesized as previously described (22).

L-Tryptophan photooxidation (18,26). A solution of 5 μ*M* Pc and 5 m*M* L-tryptophan in PBS was irradiated in a total volume of 1 mL. The irradiation system consisted of a high-intensity xenon short arc 300 W lamp model LX300F (ILC Technology, Sunnyvale, CA). Light was fed through a liquid guide fiber optic (Model 77556, Oriel Corp., Stratford, CT). The light passed through two filters, LS-700 and LL-600 (Corion, Holliston, MA), thus transmitting light over the range 600–700 nm. The fluence rate was 150 mW/cm^2 as measured with a Power-Energy meter, Model 365, with a sensor, Model 38-0101 (Scientech, Boulder, CO). The solution was kept at 4°C and bubbled with air to ensure O_2 saturation. After 2 and 4 min of illumination, 80 μL samples were collected and analyzed using HPLC to quantify the characteristic hydroperoxide products (3α-hydroperoxy-1,2,3,3α,8,8α-hexahydropyrrolo[2,3β]indole-2-carboxylic acid [HPPI] isomers) (R_t = 5.8 and 8.6 min, R_t for tryptophan = 15.7 min) derived from singlet oxygen ($^1O_2$) oxidation of tryptophan. The relative HPPI yield for each photosensitizer was measured from the HPPI chromatogram peak areas, taking the area produced by AlOHPcS$_4$ as 100%. HPLC analysis used a C18 Radial-Pak cartridge (10 × 0.8 cm) filled with 4 μm Nova Pak C18 reversed-phase packing. The peroxide isomers were eluted at 1.5 mL/min with 0.1% TFA in water and a linear gradient over 25 min from 1% to 30% methanol (0.1% TFA). The UV–visible detector was set at 280 nm.

Tryptophan oxidation was measured with solutions in PBS, 50% methanol–PBS and 1% Cremophor EL (CRM). The photosensitizers were first dissolved in PBS. Subsequently, these solutions were diluted two-fold in methanol. Alternatively, stock solutions were prepared in 10% CRM, 3% 1,2-propandiol at 50 μ*M* and diluted with PBS to the final concentration.

Cells and cell culture. A549 cells (human lung adenocarcinoma, ATCC CCL 185) were cultured at 37°C, 5% CO_2 and 95% air in RPMI 1640 media supplemented with 1% L-glutamine, 1% penicillin–streptomycin (GIBCO, Burlington, Canada) and 10% (vol/vol) fetal calf serum (FCS) (ICN, Costa Mesa, CA). The cells were harvested from 75 cm^2 T-flasks (Falcon, Mississauga, Canada) using 0.25% trypsin 1 m*M* ethylenediamine-tetraacetic acid (EDTA) (GIBCO).

Cell uptake. A549 cells were plated at a density of 1.5 × 10^5 cells per well in 24-well plates (Falcon) in growth media. Following an overnight incubation, the media were removed, the cells were rinsed 2× with PBS and overlaid with 250 μL of a 10 μ*M* Pc dilution in RPMI medium (1% FCS) with or without 4 μg/mL of human low-density lipoprotein (LDL; molecular weight 550 000) acquired commercially (Sigma, Oakville, ON, Canada). The cells were incubated for various durations up to 48 h, after which the excess dye was removed. The cells were rinsed twice with PBS and the cell lysates collected using 500 μL of 0.1 *N* NaOH. Using the Bradford method (27) in 0.1 *N* NaOH, the protein content was determined in a 50 μL sample of cell lysate. Dimethylformamide (DMF; 450 μL) and PBS (100 μL) were added to the remaining 450 μL of cell lysate in 0.1 *N* NaOH. Standard curves were made using cell lysates treated as in the foregoing, with known amounts of the appropriate Pc solution added. The cell lysates were centrifuged for 30 min at 4°C and 3500 RPM to remove any large cell debris. The dye concentration in the clear supernatant was assayed by fluorescence (F-2000 spectrofluorometer, Hitachi, Tokyo, Japan) (λ_{ex} = 608 nm, 5 nm band-pass, λ_{em} = 687 nm, 5nm band-pass). The results are expressed in nanomoles of Pc per milligram of cellular protein.

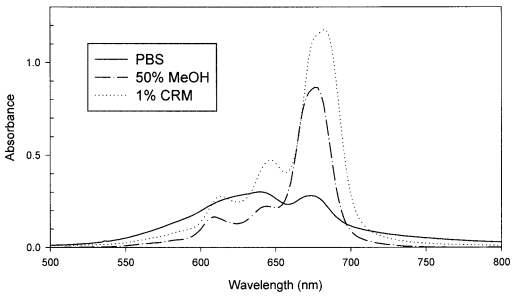

Figure 2. Absorption spectra of a 5 μM solution of $AlPcS_4(C8)$ in PBS, 50% methanol and 1% CRM.

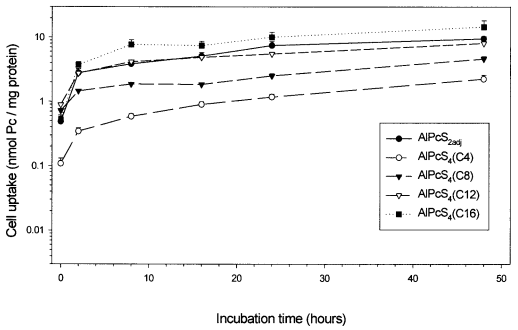

Figure 3. A549 cell membrane uptake. The A549 monolayers were incubated for up to 48 h under standard conditions with 10 μ*M* AlPc derivatives in RPMI media (1% FCS). Cell-associated dye concentration was determined using a spectrofluorometer (λ_{ex} = 608 nm, λ_{em} = 687 nm). The data are expressed as nanomoles of Pc per milligram of total protein. The mean of nine determinations is plotted, and the error bars represent the standard error of the mean.

In vitro *photocytotoxicity*. Ninety-six well microtitration plates (Sarstedt, St. Laurent, Canada) were seeded with 1.5×10^4 A549 cells and left to grow overnight at 37°C and 95% air, and 5% CO_2 in 100 μL of RPMI (10% FCS). After 18 h of incubation, the cells were rinsed twice with PBS and incubated with 50 μL of Pc at 1 μ*M* in RPMI (1% FCS) for 1 h at 37°C. Similarly cells were incubated with 50 μL of 1 μ*M* Pc supplemented with 4 μg/mL human LDL protein. After dye incubation, the cells were rinsed and illuminated according to a procedure in the literature (28–30). In brief, the cells were illuminated with red light from two 500 W tungsten–halogen lamps fitted with a filter containing aqueous rhodamine B (OD_{580} = 1.25) (Sigma, Canada). The fluence rate was 100 mW/cm^2 calculated over the Pc Q band. Using a colorimetric assay, as opposed to a colony assay, which employed the tetrazolium salt MTT (Sigma, St. Louis, MO), cell survival was assessed. A detailed account has been previously reported (11). Eight to 16 replicates were run, and experiments were repeated three times.

In vivo *PDT*. EMT-6 cells, murine mammary tumor cells, (2.5×10^5 cells suspended in 0.05 mL FCS–supplemented Waymouth medium) were intradermally implanted on the hind thighs of male Balb/c mice (20 g, purchased from Charles River, Montreal, Canada). The mice were housed in accordance with standards set by the Canadian Council on Animal Care and an in-house ethics committee. Throughout the course of the experiment food and water were supplied *ad libitum.*

Experiments were performed 1 week post–tumor implantation (tumor size 3–5 mm diameter, 2–3 mm thickness). The mice received intravenous injections of the $AlPcS_4$ derivatives suspended in PBS *via* the caudal vein, at doses as low as 0.05 to as high as 5.0 μmol/kg (0.2 mL). Mice were implanted with two tumors, one on each flank. One tumor served as an untreated control (not illuminated), whereas 24 h post–dye administration, the test tumor was illuminated for 33 min with an 8 mm diameter light ray of 650–700 nm light. The fluence rate was 200 mW/cm^2, with a total fluence per tumor set at 400 J/cm^2. A 1000 W xenon lamp (Hanovia; J. B. Atlas Inc., Rexdale, Canada) fitted with combination filters (Corion LL650 and LS700) and a 10 cm circulating water filter were used. Tumor response was assessed and tumor volumes were measured daily. The animals were evaluated over 3 weeks post-PDT, after which time if there was no palpable tumor, a complete tumor regression had been achieved. Tumor volumes were calculated by measuring the dimensions of the tumor using calipers and using a model described by O'Reilly and coworkers (31). The tumor volume is equal to the length multiplied by the width multiplied by the thickness multiplied by 0.52.

Statistical analysis. All values were expressed as mean ± standard error of the mean unless otherwise indicated. Statistical comparisons were made by one-way analysis of variance. If the level of difference was significant, then a Tukey's test was used for multiple comparisons. $P < 0.05$ was considered to be significant.

RESULTS

L-Tryptophan photooxidation

Upon excitation, Pc monomers generate $^1O_2$, which in turn photooxidizes amino acids such as L-tryptophan. L-Tryptophan oxidation yields characteristic hydroperoxide isomers, HPPI, which can be quantified using HPLC. As a result, relative $^1O_2$ yields can be inferred from such a measurement. In aqueous solution, Pc, either nonsubstituted or substituted, tend to aggregate or dimerize, which results in decreased photochemical activity (18). Pc substituted with lipophilic side chains aggregate extensively in aqueous solutions as can be seen by their absorption spectra (Fig. 2). The extent of aggregation helps determine the $AlPcS_4$ derivative's photoactivity. Table 1 is a summary of hydroperoxide formation for the Pc derivatives. The photooxidation of L-tryptophan to yield HPPI hydroperoxide isomers is greatly decreased for the Pc substituted with long lipophilic chains as compared with the parental molecule, $AlPcS_4$ (arbitrarily set at 1) when in PBS. The amphiphilic molecules are highly aggregated. However, in 50% methanol or 1% CRM the Pc monomerize, and the results are much more uniform. This is evident from the absorption spectra of a 5 μ*M* solution of $AlPcS_4(C8)$ measured in PBS, 50% methanol and 1% CRM (Fig. 2).

Cell uptake

Confluent cell monolayers were incubated for varying lengths of time with 10 μ*M* Pc in media supplemented with 1% FCS. Excess dye was removed and the cells rinsed. Subsequently, the cell lysates were assayed for Pc fluorescence. The results were calculated and expressed as nanomoles of Pc per milligram of total cell protein *versus* incubation time (Fig. 3). Cell uptake decreased in the following order: C16 > C12 > C8 > C4 with the $AlPcS_{2adj}$ derivative being similar to the $AlPcS_4(C12)$ derivative. Chromatographic mobilities of the dyes correlate with the A549 cell uptake. The more lipophilic compounds, those compounds with longer retention times on the reversed-phase HPLC column (Table 1), are readily taken up by the cells to a greater extent than are the more hydrophilic compounds, $AlPcS_4(C4)$ and $AlPcS_4(C8)$. After 48 h of incubation with the $AlPcS_4(C16)$, there was a seven-fold increase in Pc uptake as compared with the C4 derivative. The uptake of the C16 derivative was significantly increased over that of the C12, C8 and C4 derivatives after an incubation of 48 h ($P < 0.05$).

Likewise, the A549 cells were incubated using the same

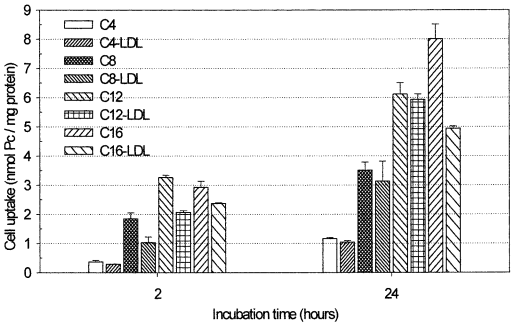

Figure 4. Effect of LDL on Pc uptake in A549 cells. A549 cells were incubated for 2 and 24 h with 10 μ*M* Pc derivative in RPMI (1%FCS) in the presence or absence of 4 μg/mL of human LDL suspension. Cell uptake was determined using fluorometry as described. The data are the means of three assays, with bars representing the standard error of the mean.

conditions; however, the Pc derivatives were suspended in RPMI media with 1% FCS and 4 μg/mL of human LDL. This corresponds to a 1000 times lower concentration of LDL than that found in whole blood (400 mg/100 mL) (32). Cell uptake of the Pc derivatives was decreased in the presence of the 4 μg/mL LDL. This was true for all $AlPcS_4$ derivatives assayed. Figure 4 is a histogram of cell uptake in nanomoles of Pc per milligram of protein *versus* time. Again, the more lipophilic derivatives have greater uptake than the more hydrophilic Pc. Assays using 40 μg/mL of human LDL completely prohibited Pc uptake (results not shown). The more lipophilic sensitizer, $AlPcS_4$(C16) derivative, displays the greatest reduction in cell uptake after 24 h incubation in RPMI media supplemented with 1% FCS and 4 μg/mL LDL. Cell uptake of the C16 derivative is significantly decreased in the presence of 4 μg/mL LDL ($P < 0.05$). At longer incubation times the presence of LDL has decreasing influence on cell uptake as the lipophilicity of the dye decreases.

In vitro photocytotoxicity

Using a constant dye concentration of 1 μ*M*, the $AlPcS_4$ cytotoxic effect on A549 cells was measured after illumination at a fluence rate of 100 mW/cm^2 calculated over the Pc Q band (660–700 nm). Cytotoxic activity varied directly with dye hydrophobicity (Table 2). Compounds having longer HPLC retention times exhibited greater photocytotoxicity, as seen by decreased values of fluence required to induce 50% and 90% cell death (LD_{50} and LD_{90}). Biological activity decreased in the following order: $AlPcS_4$(C16) > $AlPcS_4$(C12) > $AlPcS_{2adj}$ > $AlPcS_4$(C8) > $AlPcS_4$(C4).

A549 cells were incubated for 1 h with 1 μ*M* Pc diluted in RPMI media supplemented with 1% FCS. Similarly, cells were incubated using the same conditions, with 4 μg/mL of human LDL added to the media. LD_{50} values were decreased when lipoproteins were added to the media (Fig. 5). As the lipophilicity of the compound increased, the LDL effect on its cytotoxic properties was less dramatic. That is to say, the LD_{50} values for the C12 and C16 derivatives are the same, whereas the LD_{50} values for the C4, C8 and S_{2adj} compounds are greatly different with or without LDL. The addition of 4 μg/mL of human LDL significantly decreased the LD_{50} for

Table 2. Phototoxic activity of the $AlPcS_4$ derivatives compared with the chromatographic mobilities on a reversed-phase C18 HPLC column

Compound	Retention time, R_t* (min)	LD_{50}† (J/cm^{-2})	LD_{90} (J/cm^{-2})
$AlPcS_4$	8	n.a.‡	n.a.
$AlPcS_{2adj}$	24	20.4	31
C4	14	33.5	>40
C8	20	21.4	32
C12	24	11.5	24.8
C16	29	4.5	16.6

*The retention times on a reversed-phase C18 HPLC column (30 min linear gradient from 100% 10 m*M* phosphate buffer (pH 5) to 100% methanol at 1.5 mL/min). Values represent the weighted average R_t of each fraction of isomeric products of the same structural composition.

†LD_{50} is the light dose (J/cm^2) required to photoinactivate 50% of the cell population after a 1 h incubation at 37°C using 1 μ*M* of Pc in RPMI (1% FCS). LD_{90} is the light dose required to inactivate 90% of the cell population using similar conditions.

‡n.a., data not available.

$AlPcS_4$(C4), $AlPcS_4$(C8) and $AlPcS_{2adj}$. The (*) in Fig. 5 denotes a significant difference ($P < 0.05$).

In vivo PDT

Tumor response studies using the Balb/c mice with EMT-6 tumor implants demonstrated that at drug doses as low as 0.2 μmol/kg and a fluence of 400 J/cm^2, complete regression was obtained using the $AlPcS_4$ derivatives (Table 3). Cure rates of 75%, 80%, 67% and 50% were obtained for the C16, C12, C8 and C4 derivatives, respectively, at 0.2 μmol/kg. The parental molecule, $AlPcS_4$, was not active at a dose of 5 μmol/kg (29,30). $AlPcS_{2adj}$, a known amphiphilic Pc, was active at a dose of 1 μmol/kg, with a cure rate of 87%. Post-PDT edema was significant using all the $AlPcS_4$ derivatives,

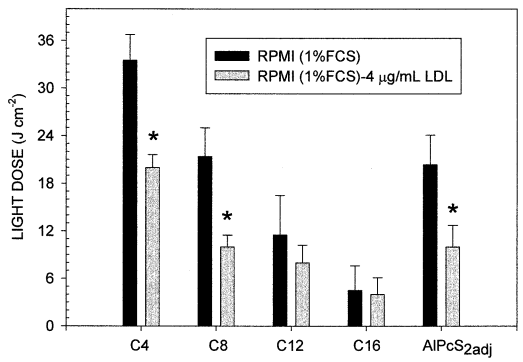

Figure 5. A549 photocytotoxicity. Effect of light dose on A549 cell survival. Cells were incubated for 1 h with 1 μ*M* Pc in RPMI (1% FCS) (black bar) or in RPMI (1% FCS) with 4 μg/mL of human LDL (gray bar). The % cell survival was determined *via* an MTT assay and plotted on a semilog scale. The light dose required to photoinactivate 50% of the cell population (LD_{50}) was extrapolated from each curve. The LD_{50} values for each Pc derivative are plotted. The error bars represent percent standard error of the mean. The data are the means of eight samples per experiment. The assay was repeated a minimum of three times. The (*) denotes significant differences observed in LD_{50} values for each compound with or without the addition of LDL to the incubation medium.

Table 3. Tumor response in Balb/c mice transplanted with EMT-6 tumor cells

Compound	Dose (μmol/kg)	Number of mice (*n*)	Edema*	Tumor necrosis†	Tumor regression‡
$AlPcS_{2adj}$	1	8	++++	8	7
$AlPcS_4$ (27)	5	4	++++	3	0
C4	0.2	2	+++	2	1
	0.5	4	++++	4	1
	0.75	6	++++	5	2
	1.0	4	++++	2	0
C8	0.1	4	++	0	0
	0.2	6	++	2	2
	0.5	6	+++	6	5
C12	0.1	4	++	4	0
	0.2	10	+++	10	8
	0.5	4	++++	4	4
	2.0	3	++++	3	3
C16	0.05	4	+	1	0
	0.1	4	+++	3	0
	0.2	4	++++	4	3
	0.5	4	++++	4	4
	1.0	3	++++	3	3

*Edema was evaluated 24 h post-PDT and defined as marginal (++), extensive (+++) or severely swollen, affecting the muscle (++++).

†Necrosis is identified as the appearance of flat and necrotic tissue within 2–3 days post-PDT as observed macroscopically.

‡Regression is defined as the absence of a palpable tumor 3 weeks post-PDT.

with the legs being severely swollen and the muscle affected. The edema was transient and subsided within 48 h post-PDT, with no permanent leg paralysis. None of the Pc derivatives were toxic, with no mortality being observed.

DISCUSSION

PDT using Pc has been well documented (7,8). Pc are promising PDT agents because of their favorable photochemical and photophysical properties and the ability to change their properties, such as solubility, through the addition of substituents to the periphery of the macrocycle. Amphiphilic sensitizers, those having both hydrophilic and hydrophobic moieties, have been shown to be more photodynamically active both *in vitro* and *in vivo* than are sensitizers that are strictly one or the other (11,12). The amphiphilic character promotes cell uptake and better intracellular targeting to more photosensitive sites (17). A number of *in vitro* and *in vivo* studies to evaluate the potential usefulness of amphiphilic photosensitizers as anticancer agents have been conducted (11–14,16,33,34).

In this study, the amphiphilicity of $AlPcS_4$ was modified *via* the addition of alkyl chains of varying length through a sulfonamide bond. Using a similar methodology, caproic acid side chains have been added to $AlPcS_4$, and these $AlPcS_4$ derivatives have recently been coupled *via* a carbodiimide method to various protein carriers to improve upon Pc delivery to target tissue (28–30). Brasseur *et al.* have reported using maleylated bovine serum albumin–Pc conjugates to increase uptake of the dye through the scavenger receptor of macrophages with promising results (28). LDL was also covalently labeled with $AlPcS_4$ (30). Marginal success was obtained using this conjugate as compared with Pc inserted noncovalently into the phospholipid bilayer *via* long alkyl chains similar to those investigated in the present study. Monoclonal antibodies such as anti-CEA (carcinoembryonic antigen) (35) and anti-CD3+ (36) have similarly been covalently coupled to $AlPcS_4$ *via* the six-carbon chain. In both cases, cell uptake of the Pc was greater using the antibody–Pc conjugate. The same principal was applied to adenovirus capsid proteins to target the integrin receptors found in abundance on cancer cell lines. Again, cell uptake of the $AlPcS_4$–protein was enhanced; however, photocytotoxicity was not. This was most probably because of increased aggregation of the bioconjugate resulting in poor $^1O_2$ yields (29).

The present study evaluates a series of $AlPcS_4$ substituted with lipophilic chains of varying lengths on the macrocycle. The absorption spectrum of a monomerized Pc encompassing both the Soret and Q bands is depicted in Fig. 1 along with the chemical structure. The retention times (R_t) of these $AlPcS_4$ derivatives were determined on a reversed-phase C18 column and served as a measure of their relative hydrophobicities. In addition, L-trytophan photooxidation, indicative of $^1O_2$ production, was measured using three different conditions: PBS, 50% MeOH and 1 % CRM (Table 1). $^1O_2$ yield is inversely proportional to the extent of sensitizer aggregation (18,28). Aggregation is dictated in large part by the ring substituents and the axial ligands on the central metal ion. Monomeric Pc molecules with identical central metal ions and varying degrees of sulfonation retain the same photochemical activity (18). Pc aggregation is easily detected spectroscopically with a 30–50 nm blueshift of the Q band absorption peak, which appears more broad and less intense (28). Upon excitation with light, aggregated photosensitizers dissipate their energy through internal conversion to the ground state rather than through triplet formation with subsequent $^1O_2$ production (5,18). Hydroperoxide formation was greatly decreased when measured in PBS and varied inversely with the retention time (Table 1). HPPI formation was greater for compounds with shorter retention times. The more lipophilic compounds aggregated more readily in aqueous solution, thus reducing their photochemical activities. When HPPI yields were measured in 50% methanol, the results were more uniform because the photosensitizer becomes more monomerized (Fig. 2). L-Tryptophan oxidation produced by the $AlPcS_4$ long chain derivatives approached that of the parental $AlPcS_4$ molecule. However, when the Pc derivatives were solubilized in 1% CRM, HPPI formation after irradiation of the C4, C8, C12 and C16 derivatives of $AlPcS_4$ surpassed that of $AlPcS_4$. Spectroscopically, the lipophilic compounds were monomerized in 1% CRM (Fig. 2), thus increasing their photochemical activities, whereas the $AlPcS_4$ was less monomeric (Table 1). Similar studies using a series of amphiphilic silicon Pc substituted with charged axial ligands gave high yields of hydroperoxide isomers compared with $AlPcS_4$, presumably on account of decreased aggregation because the axial ligands prevent stacking of the hydrophobic Pc core (personal observation). A silicon Pc bearing a long chain amino axial ligand has shown promise as a PDT agent and is about to enter clinical trials (7,37). Presumably, the potential of this photosensitizer is at

least partly because of the axial ligand preventing aggregation of the chromophore.

The results of the L-tryptophan photooxidation assay indicated that the lipophilic $AlPcS_4$ derivatives substituted with long carbon chains can indeed produce $^1O_2$ upon irradiation. This, however, is not necessarily indicative of their photodynamic potential. Many biological factors play key roles in determining the usefulness of a sensitizer in PDT, most notably cell uptake, subcellular localization, selective tumor accumulation and degree of sensitizer aggregation (9,38). To further elucidate, in the present study, A549 cell uptake of the AlPc derivatives was measured over time. Cell uptake varied directly with the degree of hydrophobicity. Following a 48 h incubation with the dye, the A549 cells took up seven-fold more C16 compound than C4 compound. Cellular uptake increases with the lipophilicity because of the better membrane penetrating properties of the sensitizer. In comparison, $AlPcS_{2adj}$, having a retention time similar to that of the $AlPcS_4$(C12) molecule, exhibited similar cell uptake properties (Fig. 3). Margaron *et al.* reported similar findings using a series of zinc Pc, where the most lipophilic sensitizers exhibited the highest cell uptake, whereas an iodinated ZnPc entered the cells to a lesser extent because of low affinity for the plasma membrane (11,13). Findings of this nature have also been reported using a series of gallium Pc, where a six-fold increase in cell uptake of the dye was measured using a disulfonated gallium Pc substituted with two adjacent *t*-butyl groups on the macrocycle (39,40). Comparable studies using a series of $AlPcS_{1-4}$ compounds found the more hydrophobic dyes accumulated to a greater extent intracellularly than the hydrophilic dyes (41).

Serum proteins greatly influence dye uptake by the cells *in vitro* and *in vivo. In vivo,* serum proteins are predominantly responsible for the transport of photosensitizers throughout the body. Serum albumin generally transports hydrophilic photosensitizers, whereas more hydrophobic sensitizers localize preferentially in lipoproteins, leading to enhanced intracellular accumulation of the dye *via* receptor-mediated endocytosis (38,42–44). *In vitro,* serum proteins have been reported to compete with cells for ZnPc derivatives, thus decreasing cell uptake of the dye (45). A compound binding tightly to albumin will remain bound to the protein and be metabolized as such or will be transferred to a cell binding site with a greater affinity for the dye than for albumin. It has been reported that association of porphyrins to BSA inhibits cellular uptake of the dye because of the strong interactions between the dye and the albumin (5).

The A549 cells were incubated with 10 μ*M* of the $AlPcS_4$ derivatives in the presence of human LDL. When 40 μg/mL LDL was used, no cell uptake of the dye was observed. However, when cells were incubated under the same conditions with 4 μg/mL LDL, cell uptake of the dye was reduced as compared with when the cells were incubated with dye in media (1% FCS) only (Fig. 4). Although there is decreased uptake of all the Pc derivatives in the presence of LDL after a 24 h incubation, this decrease in $AlPcS_4$(C16) cell uptake is most pronounced. The LDL, presumably because of increased protein–dye interaction (5), profoundly affects the most lipophilic compound (C16).

In vitro photocytotoxic activities of the $AlPcS_4$ compounds were determined using the colorimetric MTT assay. This quick and reliable assay has been shown to correlate well with other cell viability tests including the clonogenic assay in the case of PDT inactivation of cells using benzoporphyrin derivative monoacid ring A (BPD-MA) and hematoporphyrin derivative (HpD) (11). Figure 5 is a plot of the LD_{50} values for each dye in media (1% FCS) with or without the addition of 4 μg/mL LDL. Cytotoxic activities varied directly with retention times regardless of LDL involvement (Table 2, Fig. 5). Amphiphilic sensitizers are more photodynamically active because presumably they localize at protein surfaces and membrane interfaces (9,10). $AlPcS_4$(C16) was seven times more active than $AlPcS_4$(C4) in the absence of lipoproteins. However, this difference is less pronounced when 4 μg/mL of LDL was added to the incubation. Interestingly, although there was a reduction in cell uptake of the $AlPcS_4$(C16) compound when 4 μg/mL LDL was present, there is no difference in cytotoxic activity. In addition, the $AlPcS_4$(C4) dye exhibited similar cell uptake properties regardless of the addition of LDL to the incubation media, whereas there is an important difference in cytotoxic action in the presence of LDL. LD_{50} values of 33.5 and 20 J/cm^2 were measured for the C4 derivative in the absence or presence of 4 μg/mL LDL, respectively. The cell uptake is not affected in the presence of LDL for the C4 derivative. The cytotoxicity is enhanced in the presence of LDL; thus, the LDL must shuttle the C4 derivative to photosensitive sites on or within the cell. Thus, we see the decrease in light dose required to inactivate A549 cells. Similarly, despite greatly decreased cell uptake in the presence of LDL, the C16 toxicity was unaffected, presumably because of LDL shuttling of the photosensitizer to sensitive sites.

The role of the LDL receptor pathway in the delivery of lipophilic sensitizers is well documented (46–49). After sensitizer internalization, the dyes accumulate in lipophilic compartments including the plasma and organelle membranes (38). Recently, in a study where the $AlPcS_4$(C12) derivative was noncovalently inserted into the phospholipid moiety of the LDL, *in vitro* photocytotoxicity results were improved over the $AlPcS_4$(C12) alone (30). However, *in vivo* the $AlPcS_4$(C12) noncovalently coupled to the LDL or the $AlPcS_4$(C12) alone exhibited similar cytotoxic activities. In the blood stream, the lipophilic sensitizer naturally redistributes to the LDL molecules and is taken up by the cells *via* the LDL receptor route (38). On the contrary, a study using BPD-MA either associated with lipoproteins or not, found the BPD-MA–LDL conjugate had enhanced tumoricidal activity as compared with the sensitizer alone (50,51).

In vitro results were promising; however, *in vivo* results were more impressive. At dye doses of 0.2 μmol/kg, complete tumor regression of the EMT-6 tumor model was observed for the C16, C12 and C8 derivatives of $AlPcS_4$ (Table 3). $AlPcS_4$(C16) was found to be the most effective. Complete tumor regression at 5 μmol/kg of the parental $AlPcS_4$ was not observed. At drug doses as high as 2 μmol/kg (10 times more than required for tumor regression), there was no mortality or permanent leg paralysis caused by extensive muscle damage.

In summary, our data show that $AlPcS_4$ derivatives monosubstituted with carbon chains of varying lengths are potent photosensitizers both *in vitro* and *in vivo.* L-Tryptophan pho-

tooxidation, indicative of $^1O_2$ production upon illumination, surpassed that of the parental $AlPcS_4$ molecule when the lipophilic compounds were in the appropriate environment. A549 cell uptake of the dye, as well as *in vitro* photocytotoxicity, varied directly with the compound's lipophilicity: C16 > C12 > C8 > C4. Human LDL at 4 μg/mL decreased cell uptake of the Pc derivatives; however, cytotoxic potency was increased or unaffected. This is seemingly because of better intracellular targeting. At low drug doses, 0.2 μmol/kg, EMT-6 tumor regression was induced. Substitution of the $AlPcS_4$ with lipophilic side chains greatly increased both the *in vitro* and *in vivo* cytotoxic potency of the photosensitizer. This is further confirmation that amphiphilic sensitizers are advantageous in PDT, and studies are underway to take advantage of this (52). For instance, a series of amphiphilic trisulfonated Pc bearing long aliphatic chains on the fourth isoindoline has been synthesized and is presently being evaluated as photosensitizers with some promising results (53).

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