Photocytotoxic efficacy of sulphonated species of aluminium phthalocyanine against cell monolayers, multicellular spheroids and in vivo tumours

W.S. Chan¹², C.M.L. West*, J.V. Moore* & I.R. Hart¹

¹Biology of Metastasis Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC2A 3PX; ²Radiobiology Department, Paterson Institute for Cancer Research, Christie Hospital and Holt Radium Institute, Wilmslow Road, Manchester M20 9BX, UK.

Summary The problem of relying solely on in vitro data to predict photosensitiser efficacy was demonstrated by examining H; take up and the ability to mediate photocytotoxicity of mono-, di-, tri- and tetra-suphoniated species of chloroaluminium phthalocyanine (AlS₃Pc) in monolayer cultures of murine Colo 26 cells and in both monolayer and spheroid cultures of human WiDr cells. Cells treated in vitro, whether in monolayer or as spheroids, with the less sulphonated derivatives, AlS₃Pc and AlS₄Pc, were more susceptible to photocytotoxicity than those treated with AlS₄Pc, cells treated with AlS₄Pc were even less susceptible to the cytotoxic effects of light irradiation. Generally these results mirrored the cellular uptake in vivo. When WiDr spheroids were increased in size from 250 μm to 500 μm there was a reduction in uptake of AlS₃Pc and AlS₄Pc which was reflected by the decreased sensitivity of the larger spheroids to the effects of light irradiation. AlS₃Pc had no effect against Colo 26 cells growing as s.c. tumours in syngeneic BALB/c mice; whereas AlS₃Pc, AlS₄Pc and AlS₅Pc produced significant reductions in tumour weights 5 days post laser light irradiation. Of these, AlS₄Pc had the most dramatic effect on the colony forming efficiency of tumour cells recovered 24 h after PDT. While, despite their effects on tumour size, AlS₄Pc and AlS₅Pc scarcely affected the subsequent viability of cells from dissociated tumours. Thus the in vitro efficacy of the sulphonated species of phthalocyanines is not necessarily predictive of their in vivo effectiveness.

The metallophthalocyanines (MPc) are good candidates for photosensitizers which may prove effective in the PDT of cancer (Spikes, 1986; Ben-Hur, 1987; van Lier et al., 1987). One of the major advantages of the MPc is that this group of compounds absorbs light strongly in the red region (Q band – 670 nm); a region of the spectrum which permits good tissue penetration. Generally, the active MPc contain central diamagnetic metal ions, such as aluminium, gallium, tin and zinc (Brasseur et al., 1985; Ben-Hur & Rosenthal, 1986; Brasseur et al., 1987; Chan et al., 1987a, 1987b; Brattle et al., 1988; Paquette et al., 1988) while sulphonation of the benzene rings of the macrocycle leads to solubility in water which facilitates administration to animals. One such compound, AlS₃Pc, has been shown by ourselves (Chan et al., 1986, 1988) and others (Sandeman et al., 1987; Tralau et al., 1987a; Nelson et al., 1988) to be a particularly promising agent for PDT. This substance possesses good tumour-localising capacity (Tralau et al., 1987a, 1987b; Chan et al., 1988, 1989) and causes substantial damage to a range of tumours of diverse histological origin (Sandeman et al., 1987; Tralau et al., 1987a; Chan et al., 1988, Nelson et al., 1988) with prolongation of survival times in treated animals (Chan et al., 1987b). Though AlS₃Pc may induce a mild immunosuppression in mice (Marshall et al., 1989) it produces much less skin photosensitivity than occurs subsequent to HpD or PIII administration which is the current clinical regimen for PDT (Tralau et al., 1989). AlS₄Pc, as originally used by us (Chan et al., 1986), was found to consist of a large number of isomers of species with varying degrees of sulphonation. The uptake and distribution of AlS₄Pc by tumour cells both in vitro and in vivo was found to be affected quite profoundly by the degree of sulphonation (Chan et al., 1990). In this paper we have sought to determine whether these differences in uptake and distribution could affect efficacy as a consequence of the balance between dye uptake and penetration, by examining the effect of the variously sulphonated species on multicellular spheroids of differing sizes and on tumours in vivo.

Materials and methods
Photosensitiser preparation
AlS₃Pc was obtained from Ciba-Geigy Dyestuffs and Chemicals (Basel, Switzerland). This material was a complex mixture of mono- to tetrasulphonated derivatives with, according to the supplier, an average of three sulphone groups. AlS₃Pc, AlS₄Pc and AlS₅Pc, individual chloroaluminium mono-, di- and tri-sulphonated derivatives, were bought from Porphyrin Products (Logan, Utah). AlS₃Pc, chloroaluminium tetrasulphonated Pc, was prepared by the condensation of aluminium trichloride with sulphophthalic acid as described (Weber & Buschel, 1962). These various derivatives were shown by spectroscopy and high pressure liquid chromatography analysis to be >90% pure though possibly containing numerous isomers (Chan et al., 1990). The various dyes were dissolved in PBS except the water-insoluble AlS₅Pc which was dissolved in 40% (v/v) ethanol/PBS for in vivo studies or in DMF for in vitro studies. The procedures of preparation and quantitation of the dye solutions have been detailed previously (Chan et al., 1990). Since the molecular weights of the individual derivatives varied, equal molarities of these derivatives were compared for biological effects.

Tumour cells
Colo 26 cells from a murine colorectal carcinoma, syngeneic to BALB/c mice, were passaged routinely in E4 growth medium containing 10% FCS (Chan et al., 1987b). These cells were used both for in vivo and in vitro experiments.

Correspondence: I.R. Hart.
*Present address: MRC Radiobiology Unit, Chilton, Didcot, Oxon OX11 0RD, UK.
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Abbreviations used: AlS₃Pc, chloroaluminium sulphonated phthalocyanine with n sulphone groups; AlS₄Pc, chloroaluminium sulphonated phthalocyanine a mixture of mono- to tetra-sulphonated derivatives. BME, Eagle’s basal medium; CFE, colony forming efficiency; DMF, dimethyl formamide; FCS, foetal calf serum; HBSS, Hank’s balanced salt solution; HpD, haematoporphyrin derivative; MPc, metallophthalocyanines; PIII, Photofrin II; PBS, phosphate buffered saline; Pc, phthalocyanine(s); PDT, photodynamic therapy.
WiDr cells from a human colon adenocarcinoma were passaged routinely in BME medium supplemented with 10% FCS (West, 1989). Multicellular spheroids of these cells were prepared by growing cultures in Petri dishes on a 1% agar base. Five days later the spheroid-containing culture supernatant was filtered to obtain homogeneously sized spheroids and the cells were grown in spinner flasks to the appropriate aggregate size as detailed elsewhere (West, 1989).

Effects of in vitro irradiation of dye-treated cells

Colo 26 cells were plated, (5 x 10^5 - 5 x 10^6) into 55 mm Petri dishes containing 5 ml growth medium and cultured for 1-3 days before being re-fed with fresh medium containing the various Pc derivatives at a final concentration of 10 μM. Following incubation in the dye-containing medium for 24 h, cells were washed three times with PBS, trypsinised, washed and counted. Cell concentrations were then adjusted and plated into triplicate Petri dishes, at cell numbers which ranged from 10^2-10^5, containing dye-free medium. Cultures were exposed to red light at doses ranging up to 2.16 J cm^-2 and then maintained in incubators for a further 10-12 days before being fixed in methanol and stained with Giemsa to examine for colony formation. Only colonies of around 50 cells or greater were counted and the CFE was determined by relating these to colony numbers on untreated control dishes (red light irradiation, 2.16 J cm^-2, but no Pc-treatment or Pc-treatment with no light irradiation) which were expressed as 100% survival.

For the various experiments reported here as well as the effects of irradiation time (light dose), the effects of Pc-dose (from 0-30 μM at a fixed light dose of 2.16 J cm^-2) and incubation period in the presence of Pc were also examined.

The red light source (600-700 nm) consisted of a bank of six fluorescent tubes filtered through a red gelatin filter. The emission spectra of this source has been documented elsewhere (Chan et al., 1986). The intensity of light measured at the cells' location was 1.2 ± 0.2 mW cm^-2 as determined by a Coherent Power Meter, Model 212 (Coherent Ltd., Cambridge, UK). These readings conflict with the value of 0.71 W m^-2 previously reported by our group (Chan et al., 1986; 1987a) but we have established that the present figures are correct and the previous values arose as a consequence of a faulty power meter.

Effect of AlS3Pc plus light on WiDr cells growing in monolayer and as spheroids

The procedure used to establish AlS3Pc-mediated cytotoxicity against WiDr cultures was similar to that described previously for PII (West, 1989). Briefly WiDr cells, in monolayer or as spheroids of 250 μm or 500 μm diameter, were incubated in growth medium containing a final concentration of 10 μM Pc derivative in spinner flasks for 24 h. Cultures were rinsed twice in PBS and disaggregated into single cell suspensions by trypsinisation. Cells were irradiated as single cell suspensions, 3-5 x 10^5 cells in 1 ml Hepes-buffered BME in 35 mm Petri dishes. Following irradiation with varying light doses up to a maximum of 15 J cm^-2 cells were then diluted to appropriate concentrations and plated into 60 mm Petri dishes containing 5 ml growth medium per dish. Cell counts were performed 20-22 days later and CFE was calculated as related to control cells (AlS3Pc exposed monolayer/spheroid cultures but no light irradiation).

In these experiments red-light was derived from a copper vapour pumped dye laser (Oxford Laser, Oxford, UK) tuned to 675 nm (laser dye, Oxazine 720). Light was delivered via a 1 mm diameter quartz fibre which projected downward (West et al., 1990) to produce an even light fluorescence (20 mW cm^-2) at the cell level as determined by a thermopile (Laser Instrumentation, Basingstoke, UK).

In vitro Pc uptake

The techniques used to determine this aspect of cell behaviour have been described in detail elsewhere (Chan et al., 1990). Briefly, WiDr cells in the exponential growth phase, either as monolayers or spheroids, were exposed to 10 μM Pc-derivative for up to 48 h and cells were then dispersed by trypsinisation. Cell numbers were determined by Coulter counter and 10^4 cells were centrifuged to form pellets. The cell pellets were digested in 0.1 M NaOH (0.1 M NaOH/ethanol for AlS3Pc) and Pc content was determined by fluorospectrophotometry at the excitation/emission wave-lengths appropriate for the specific sulphonated derivatives, as detailed previously (Chan et al., 1990).

PDT of Colo 26 tumours

The different Pc-derivatives were used to sensitize BALB/c mice bearing s.c. Colo 26 tumours which were treated subsequently with laser light irradiation as described previously (Chan et al., 1987b). Tumours were produced by injecting 10^5 cells s.c. into the flank region of female BALB/c mice (12-14 weeks old, obtained from the Imperial Cancer Research Fund Animal Breeding Unit, Clare Hall, Herts, UK). When tumours were approximately 5-7 mm diameter the mice received an injection of 0.1 ml of a 1.134 mM solution of one of the Pc-derivatives via the lateral tail vein. Twenty-four hours later PDT was performed using a copper vapour pumped dye laser (675 nm, power 50 mW). Mice were anaesthetized during PDT (Chan et al., 1987b). Light was delivered to the centre of the tumour via a 0.2 mm diameter quartz fibre (100 J/tumour) as detailed previously (Chan et al., 1987b). The tumour response to PDT was evaluated by (a) measuring tumour weight 5 days after light irradiation and (b) by determination of the number of clonogenic cells recovered from disaggregated tumour 24 h after PDT. Controls were obtained from mice where laser irradiation of tumours had been performed, but the animals had not received any dye injection; values obtained from this group were expressed as 100%. The disaggregation of tumours was achieved by incubating minced tumour in 0.02% collagenase (Type 1; Sigma Chemical Company, Poole, UK) and 0.01% DNAase (Type 1; Sigma) for 2-24 h at 37°C with continuous stirring. Cells were washed with PBS, pelleted and resuspended in growth medium and viability was determined on an aliquot by trypsin blue exclusion. From 100 to 5000 viable cells were plated into 60 mm Petri dishes (3-6 dishes per cell dose) and CFE was calculated 10-14 days later as described above.

Results

Photosensitising activity of sulphonated Pc-derivatives against Colo 26 and WiDr cells in vitro

Results from between 3-6 independent experiments are summarised in Figure 1. Increasing sulphonation was associated with diminished photocytotoxicity such that AlS3Pc and AlS4Pc were unable to reduce the surviving fraction of treated Colo 26 cells to any significant extent (Figure 1a). By contrast, at light doses of 2.16 J cm^-2, AlS3Pc and AlS4Pc reduced the surviving fraction of Colo 26 cells by greater than 99%. The relative lack of efficacy of AlS3Pc and AlS4Pc was maintained even at high concentrations of the two species (Figure 1b). Despite the fact that the AlS3Pc mixture was claimed to have an average of three sulphonate groups, its behaviour most closely resembled that of the di-sulphonate species (Figures 1a-1c).

Although the range of light doses used against the WiDr cells, both in monolayer culture and as spheroids of different size, were greater than those used in the experiments with Colo 26 cells, a similar pattern of potency was observed (Figure 2). Thus the photokilling capacity of AlS3Pc > AlS4Pc > AlS5Pc > AlS6Pc whether cells were grown as monolayer cultures (Figure 2a) or as spheroids (Figure 2b,2c).

The size of the spheroids used in these experiments (250 μm or 500 μm diameter) had an observable effect upon the response of treated cells to light irradiation (Figures 2b
and 2c). Furthermore there was a fairly marked difference between the response of cells in monolayer (Figure 2a) and as spheroids (Figures 2b and 2c). Thus the production of equivalent cytotoxicities by AlS3Pc and AlS4Pc required higher light doses in spheroids that it did in monolayer cells and it appeared that AlS3Pc and AlS4Pc were more effective against cells in the 250 \( \mu \text{m} \) aggregates than in the 500 \( \mu \text{m} \) diameter spheroids. These differences were less marked for AlS2Pc and were not apparent for AlS1Pc.

Cellular uptake of Pc derivatives in vitro

The kinetics of uptake of the various sulphonated species of AlS3Pc by WiDr cells growing in monolayer or as different sized spheroids are presented in Figure 3. The derived uptake curves are very similar to those already established for Colo 26 cells (Chan et al., 1990) and indicate that the relative amount of each individual species taken up by the cells varies considerably with AlS1Pc > AlS2Pc > AlS3Pc > AlS4Pc. The qualitative and quantitative patterns of uptake by the spheroids of the various species were remarkably similar for both sizes of spheroids (Figures 3b and 3c). It was clear however that there was a noticeable decrease in the amount of AlS1Pc taken up by spheroids, and a smaller decrease in the amount of AlS3Pc, as compared with that taken up by monolayer cells.

Anti-tumour efficacy of Pc derivatives in PDT

The effect of PDT on s.c. located growing Colo 26 tumours is illustrated in Figure 4 where the weights of tumours from the various groups, 5 days after light irradiation, are presented. It is apparent that AlS1Pc had no effect upon tumour weight but that the use of AlS3Pc, AlS4Pc, AlS5Pc or AlS6Pc all induced significant reductions (\( P < 0.001 \)) in tumour size.

Apart from the changes in total tumour weights there were also changes in the clonogenic capacity of cells recovered from treated tumours 24 h after the application of PDT (Figure 5). In this assay it can be seen that AlS1Pc and AlS2Pc were by far the most potent dyes; reducing the surviving fraction to less than 1% of that obtained from control (untreated) tumours. Because AlS3Pc had no effect on tu-
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Figure 3 Kinetics of cellular uptake in vitro of sulphonated Pc by WiDr cells a, in monolayer, b, as 250 µm spheroids or c, as 500 µm spheroids. O, AlS1Pc; ▲, AlS3Pc; □, AlS4Pc; ■, AlS2Pc. Pc concentration in the medium is 10 µM. Bars, standard deviations (s.d.). Earliest time point 1 h after addition of Pc.

Figure 4 Colo 26 tumour weights 5 days after PDT. Tumour-bearing mice were allotted to one of six groups: mice received a, PBS; b, AlS1Pc; c, AlS3Pc; d, AlS4Pc; e, AlS2Pc; f, AlS3Pc. Individual species of dye (0.1 ml of 1.135 mm) were injected i.v. via tail vein 24 h prior to PDT. ■, anaesthesia but no laser irradiation; ▲, laser light irradiation. Numbers at base of columns represent individual tumours per group. Bars, s.d. of mean tumour weights.

Figure 5 Clonogenic capacity of Colo 26 cells recovered from tumours after the same PDT treatment as described in Figure 4. Cells recovered from tumours of animals which had received, a, AlS1Pc; b, AlS3Pc; c, AlS4Pc or d, AlS2Pc. Number of colonies per gram of tumour obtained from control mice (no dye injection but with laser irradiation) are expressed as 100% survival. Bars, s.e.

Discussion

The results presented in this study show that the degree of sulphonation of chloroaaluminium phthalocyanine can have a profound effect upon light-induced cytotoxicity in vitro and in vivo. Thus against cells and spheroids in tissue culture it is the least sulphonated species which are the most phototoxic (Figures 1 and 2). Conversely when Colo 26 tumours in vivo were subjected to PDT protocols (Chan et al., 1987b) it was apparent that AlS2Pc, a highly potent photosensitiser in vitro, had virtually no cytotoxic capacity (Figure 4). These results appear to be due, partially at least, to the relative differences in uptake that exist between cells in tissue culture or in vivo (Chan et al., 1990). Since the degree of sulphonation is correlated inversely to the lipid/water partition coefficient (Berg et al., 1989a) an increase in the number of sulphonation groups would lead to a decrease in Pc lipophilicity. The greater the partition coefficient, the higher the concentration of the drug in the membrane and the faster the rate of diffusion into the cell (Benet & Sheiner, 1985). In accord with this possibility the less sulphonated Pc are taken up to a greater extent, and at a faster rate, than the more sulphonated Pc in tissue culture cells (see Chan et al., 1990; and Figure 3). This in vitro correlation exists with WiDr cells whether they are growing in monolayer culture or as spheroids of two different sizes. AlS1Pc and AlS4Pc are taken up to lesser extent by cells in spheroids than by cells in monolayer culture (Figure 3) and this reduced accumulation in spheroids is reflected in the increased light dose required to achieve comparable levels of cell killing (Figure 2). It appears from our experiments in tissue culture that observed cytotoxicity mainly is a consequence of photosensitiser uptake where decreased sulphonation is correlated with increased phototoxic efficacy. While these data agree with those obtained with other phthalocyanines, GaS2Pc for example (Brasseur et al., 1987), they are in conflict with others findings on ZnS2Pc and even AlS2Pc (Brasseur et al., 1988; Berg et al., 1989). The reason for these discrepancies is not known but may relate to either differences in purity or isomer content of the photosensitising agents. Also, it has been proposed that disulphonated material where the two sulphonate groups are adjacent may be more effective than material where the sulphonate groups are on opposite sides of the molecule (Paquette et al., 1988). Direct comparison of our results with others will require further work to characterise both the isomer content and the position of the sulphonate groups.

It had also been shown that the amount of PII, a more hydrophobic sensitisier (Berg et al., 1989a), taken up by spheroids diminished markedly with increasing aggregate size, as did resultant phototoxicity (West, 1989). Thus the less hydrophilic Pc derivatives used in this study, AlS2Pc and AlS3Pc, showed a reduction in accumulation in WiDr cells...
Growing as spheroids relative to monolayer cells, and the differences for AlS₄Pc were similar (Figure 3) to those obtained with PII (West, 1989). These findings suggest that the contrast in Pc-derivative accumulation and retention observed in the in vitro and the in vivo situation (Chan et al., 1990) may be attributable partially to lower penetration into three-dimensional, as compared to two-dimensional, tumour foci by hydrophilic species. We have shown previously that Colo 26 growing s.c. in BALB/c mice accumulated photosensitiser to a greater extent as the degree of sulphonation increased so that AlS₄Pc > AlS₃Pc > AlS₂Pc > AlS₁Pc (Chan et al., 1990). We now show that AlS₄Pc is ineffective in the PDT of s.c. tumours whereas the other Pc derivatives can reduce substantially the size of treated tumours (Figure 4) or the clonogenic capacity of component neoplastic cells (Figure 5). Others have found that the more hydrophilic, AlS₄Pc and AlS₃Pc, derivatives localise mainly in the extracellular stromal compartment of the tumour (Peng et al., 1990a, 1990b). This suggests that one possible reason for the relative inefficiency of AlS₄Pc compared with AlS₃Pc may be that, though the latter derivative is taken up to a lesser extent, what is accumulated is localised in an area where it is able to exert maximum effect. Other groups have also shown AlS₄Pc to be a more effective photosensitiser than HpD (Canti et al., 1990).

We have suggested that a possible explanation for the dearth of AlS₄Pc in tumour tissue may be the efficient removal of this dye from the circulation by hepatic accumulation and retention (Chan et al., 1990). The effects of the sulphonated species on tumour weight and clonogenic cells (Figures 4 and 5) show some discordancy. Thus AlS₄Pc and AlS₃Pc evoke significant reductions in tumour size (Figure 4) but have little effect on the CFE of cells from the disaggregated tumour (Figure 5). It may be that the gross weights of tumours provides an inaccurate assay relative to the CFE assay. Alternatively AlS₄Pc and AlS₃Pc have been stated to exert a greater effect against tumour vasculature (Henderson & Farrell, 1989; Peng et al., 1990c) and their mode of cell killing may differ from that of the more potent AlS₃Pc. Henderson and Farrell (1989) claimed that an enriched fraction of the mono-sulphonated species was the most effective photosensitiser in vivo compared to the other sulphonated species. However since these workers were able to dissolve the dye in PBS, it is possible that the effect they observed might have been due to the presence of different isomers or additional sulphonated species (Henderson & Farrell, 1989). Such a possibility would explain the differences from our results.

Whatever the relative role or importance of this type of pharmacokinetics and intra-tumoural location of Pc-derivatives it is clear from our studies that a thorough understanding of not only the in vitro characteristics of Pc dyes but also an appreciation of their in vivo behaviour is required before selection of the optimal component for PDT can be made.

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