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CARFAX

ORIGINAL ARTICLE

Subcellular distribution and photocytotoxicity of aluminium phthalocyanines and haematoporphyrin derivative in cultured human meningioma cells

G. M. MALHAM,[1] R. J. THOMSEN,[2] G. J. FINLAY[3] & B. C. BAGULEY[3]

[1]*Department of Neurosurgery, Auckland Hospital and Departments of* [2]*Pathology and* [3]*Cancer Research Laboratory, University of Auckland School of Medicine, New Zealand*

Abstract

The photocytotoxicity characteristics of aluminium phthalocyanine chloride (AlPc), aluminium phthalocyanine disulphonate (AlS_2Pc), aluminium phthalocyanine tetrasulphonate (AlS_4Pc) and haematoporphyrin derivative (HpD) were compared using primary cultures of human meningioma cells. Cells were preincubated with the photosensitising agent for 16 h, then illuminated for 15 min with broad band red light (5 $\mu W/cm^2$). The resultant cytotoxicity was assessed by tetrazolium (MTT) reduction 24 h later. AlPc was found to be 400, 10,000 and 250 times more potent that AlS_2Pc, AlS_4Pc and HpD, respectively, as an *in vitro* photosensitizing agent for meningioma cells. The subcellular localisation of AlPc, AlS_2Pc, AlS_4Pc and HpD in meningioma cells was determined by confocal laser scanning microscopy. None of the agents localized to the nucleus. The distribution of AlPc was quite diffuse through the cytoplasm. In contrast, AlS_2Pc and AlS_4Pc were localized in vesicles suggestive of lysosomes, and HpD in membranous organelles distinct from mitochondria. AlPc and HpD were tested with five different meningioma samples and provided a range of IC_{50} values from 0.009 to 0.022 μM and from 3.5 to 6.5 μM, respectively. When the MTT assay with AlPc was performed 0, 24, 48 and 72 h after illumination, the mean IC_{50} values were 0.25, 0.037, 0.019 and 0.012 μM, respectively, indicating that the cytotoxic effect continued to increase up to 72 h. Cells were incubated with AlPc and HpD for different times up to 24 h before exposure to light. AlPc cytotoxicity was half-maximal with an incubation time of 8 h, whereas HpD cytotoxicity was half-maximal with an incubation time of 2 h, implying slower uptake kinetics for AlPc than for HpD. These data indicate unique features of AlPc which suggests its application as a potent, non-toxic photosensitizer in the photodynamic therapy of human meningiomas.

Key words: *Confocal microscopy, haematoporphyrin derivative, meningioma, photodynamic therapy, phthalocyanine, tissue culture.*

Introduction

Meningiomas are the most frequently occurring benign CNS tumours.[1] The treatment of choice is complete surgical removal, but this is not always possible with meningiomas which are invasive, *en plaque* or densely adherent to critical structures such as blood vessels and cranial nerves.[2] Recurrent meningiomas may necessitate multiple neurosurgical procedures and lesions which are subtotally excised or malignant require adjuvant therapy.[3] External beam irradiation may be associated with considerable morbidity including necrosis of normal brain tissue, visual loss, pituitary dysfunction or radiation induced neoplasm.[4] Hormonal manipulation by oestrogen and progesterone therapy to retard meningioma growth requires prolonged treatment and results have been controversial.[5] Thus, conventional adjuvant therapy for recurrent, malignant and incompletely excised meningiomas is suboptimal, and photodynamic therapy (PDT) offers an alternative treatment.

PDT is a tumour treatment modality where cell death is initiated by the photoactivation of a tumour localising photosensiizing agent. The drug is inert

Correspondence to: Dr Gregory M. Malham, Department of Neurosurgery, Auckland Hospital, Private Bag 92024, Auckland, New Zealand.

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until illuminated by light of an appropriate wavelength.[6] Photoactivation of the agent results in the production of singlet oxygen or free radicals, the objective of PDT is to induce selective tumour cytotoxicity with minimal systemic side-effects. The most widely used photosensitizing agent since its discovery in 1960 has been haematoporphyrin derivative (HpD), commonly known as Photofrin I, which has been investigated in malignant neck, breast, lung oesophageal, gastric, colon and bladder cancer.[7] This agent exhibits dose-dependent in vitro cytotoxic activity against meningioma[8] and has been employed in the treatment of malignant brain tumours such as astrocytomas.[9]

One disadvantage of HpD is that it is composed of a heterogeneous poorly defined mixture of related molecules. Moreover, it is photoactivated at short wavelengths (Soret peak 390 nm; minor peak 630 nm) which are suboptimal for tissue penetration, and has the side effect of prolonged skin photosensitivity.[10] An aluminium-chelated phthalocyanine derivative, aluminium phthalocyanine chloride (AlPc) is advantageous in being easily synthesised, inexpensive, commercially available and composed of a single molecular species. AlPc has a broad band absorption spectrum (600–800 nm) which is convenient for PDT[11] because of the excellent tissue penetration of light in this wavelength range.[12] The disulphonated[13,14] (AlS_2Pc) and tetrasulphonated[15–18] (AlS_4Pc) derivatives of AlPc are more water-soluble, with a well defined absorption peak at 677 nm[12] and have been extensively studied *in vivo*.

In this study, we have compared the photocytotoxic efficacy of AlPc, AlS_2Pc, AlS_4Pc and HpD against pure populations of benign human meningioma cells in culture[19,20] and have also compared the subcellular distribution of these drugs using confocal laser scanning microscopy.

Materials and methods

Photosensitizing agents

HpD was purchased from Sigma Chemical Company (St Louis, MO, USA) and an 8 mM stock solution prepared by dissolution in 0.1 M sodium hydroxide (NaOH) with pH adjusted to 7.2 using 0.1 M hydrochloric acid (HCl) and filter sterilized. AlPc was obtained from Eastman Kodak Company (Rochester, NY) and a 5-mM stock solution prepared by dissolution in dimethylsulphoxide (DMSO). AlS_2Pc was kindly provided by Dr G. Halbert, Cancer Research Campaign (Strathclyde, UK) and AlS_4Pc was purchased from Porphyrin Products Inc. (Ogden, Utah). Stock solutions of both (5 mM) were prepared by dissolution in phosphate buffered saline (PBS). All four solutions were stored at $-20°C$ in the absence of light.

Cell culture

Meningiomas removed neurosurgically from five patients were studied. Tumour specimens were collected in culture medium and disaggregated the same day by mincing with crossed scalpel blades followed by enzymatic digestion with pronase (1 mg/ml Calbiochem), collagenase (1 mg/ml; Sigma Chemical Co. *Clostridium histolyticum*, crude), and DNAase (1 mg/ml; Sigma, type 1, pancreatic, crude). Cells were centrifuged to remove debris and proteases, washed twice and seeded into separate culture flasks. The cells were grown to confluence and maintained in an alpha modified minimal essential medium (α-MEM; Gibco, New Zealand) containing 20% heat inactivated fetal calf serum (FCS; Gibco), penicillin (100 U/ml; Sigma) and streptomycin (100 μg/ml; Sigma) at 37°C in a humidified 5% CO_2 atmosphere. Growth medium was changed every 7 days. Cells were passaged by detaching them from the plastic using trypsin (0.07% w/v; Difco) in citrate saline solution (trisodium citrate dihydrate 4.4 g/l, KCl 10 g/l, pH 7.3). Identification of cultured cells as meningioma in previous studies has been established by light microscopic, electron microscopic and chromosomal studies.[20] For experiments, the cells were trypsinized, resuspended in fresh medium, washed and plated into 96-well trays at 10^4 cells per well (150 μl/well) within one to two passages from explanting. Cultures were incubated for 24 h, after which the medium was aspirated and 150 μl of phenol red-free medium (α-MEM, Gibco) containing 20% FCS and either HpD (0.3, 1, 3, 10 or 30 μM) or AlPc (0.01, 0.03, 0.1, 0.3 or 1μM) was added to each well. Each plate contained wells to which photosensitising agent was not added to serve as controls. Except where indicated, cells were incubated with respective agent for 16 h prior to photo-irradiation.

Photo-irradiation

Photo-irradiation was carried out using a locally constructed support for a plastic 96-well microculture plate which was placed on the primary focusing element of a 300-W overhead projector. The converging beam from the projector illuminated the lower surface of the 96-well plate at a height of 25 cm from the focusing plate. At this distance, the intensity was found to vary by less than $\pm 15\%$ over the central 24 wells used in the experiment (using a

Newport research model 815 power metre). The uniformity of the illumination was confirmed by a cytotoxicity assay. The light was also passed through a red filter fitted to the plate support to reduce the total radiant intensity. The red filter in the plate support transmitted only those wavelengths greater than 600 nm produced by the tungsten source, sufficient to cover the entire significant absorbing region of each agent. The 96-well plate was placed in a bath of ice water which permitted less than a 1°C temperature variation in the cultures over the course of 15 min light exposure. The temperature was monitored by a thermocouple probe (Digitron model 3208K). The total light intensity at the plate was 300 mW/cm^2 but much of this was in the infra red. The intensity at the plate after passing through a 650 ± 20 nm filter (Melles-Griot, Irvine, CA) was 5 mW/cm^2 (± 15%).

Cytotoxicity assays

After photo-irradiation, the medium was aspirated and fresh α-MEM/FCS was added. The surviving cell population of each plate well was assessed by the MTT {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma} assay,[21,22] MTT was dissolved in PBS at 5 mg/ml, filter-sterilized and stored at −20°C in the absence of light. MTT solution was added to each well (15 μl/well) either immediately, 24, 48 or 72 h following light exposure. The plates were incubated at 37°C for a further 2 h to allow MTT metabolism, after which the medium was aspirated and the formazan crystals produced by viable cells were dissolved by the addition of DMSO (100 μl/well). Control groups received identical concentrations of photosensitizing agent and MTT without photoactivation. Absorbance was measured using a microplate reader (Dynatech MR 600) and results expressed as a percentage of control values. Experiments were performed using quadruplicate cultures and standard errors were typically < 15% of mean values. Unless stated otherwise, cells were left in drug-containing medium during illumination.

Confocal microscopy

A Leica TCS 4D confocal laser scanning microscope was used to assess the distribution of AlPc, AlS_2Pc, AlS_4Pc and HpD in meningioma cells following incubation with the agents for 16 h. An argon/krypton laser was used with a Rhodamine/Texas Red filter set (568 nm excitation, > 690 nm emission). Although not optimal, the 568 nm laser was adequate to obtain excitation of all four agents. Single optical slices were taken using a 63 × 1.4 NA objective. Images were stored as TIFF files and recorded on an Agfa PCR II film recorder.

Results

The *in vitro* cytotoxic activities of the AlPc, AlS_2Pc, AlS_4Pc and HpD as photosensitizing agents were compared. AlPc was found to be 400, 10,000 and 250 times more potent than AlS_2Pc, AlS_4Pc and HpD, respectively, as an *in vitro* photosensitizing agent (Fig. 1). AlPc and HpD were tested in greater detail on five individual meningioma cultures. Except for one sample, the IC_{50} values fell in the range 0.009–0.022 μM for AlPc and 3.5–6.5 μM for HpD, respectively (Table I). The remaining sample was approximately 4-fold more resistant to each agent. In the absence of illumination, AlPc was not toxic to any of the meningiomas studied up to a concentration of 1 μM, which was 50-fold greater than the IC_{50} value (Fig. 2).

The effect of preincubating cells with AlPc and HpD before exposure to light was investigated. Both agents were added to cultures 2, 4, 8, 16 or 24 h before photo-irradiation. The drugs differed in their dependency on preincubation time. AlPc cytotoxicity was half-maximal with an incubation time of 8 h, whereas HpD cytotoxicity was half-maximal with an incubation time of 2 h (Fig. 2).

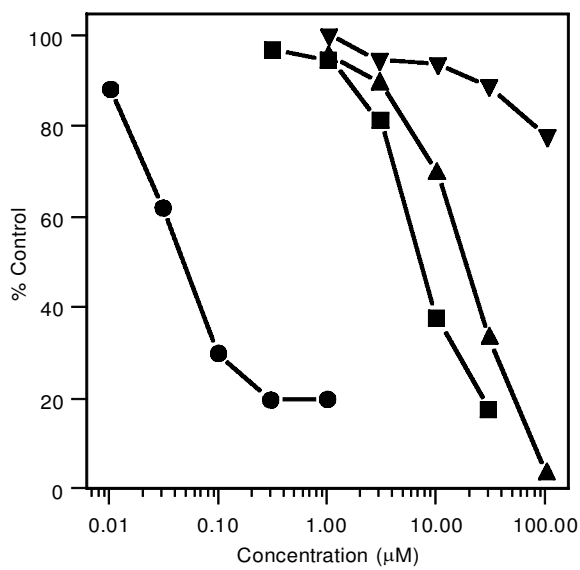


FIG. 1. Photocytotoxic effect (percentage of control) of AlPc (●), AlS_2Pc (▲), AlS_4Pc (▼) and HpD (■) incubated with meningioma cells for 16 h prior to illumination. Values are expressed as percentages of those obtained with cultures incubated in the absence of drug. Results for AlPc and HpD are mean values from three meningiomas.

TABLE I. IC_{50} values for HpD and AlPc following photoirradiation for 15 min

Meningioma	HpD (μM)	AlPc (μM)
1	4.4	0.022
2	3.5	0.009
3	5.2	0.017
4	6.5	0.009
5	23.2	0.12

The effect of replacing the culture medium with drug-free medium prior to illumination was compared for AlPc and HpD. Removal of extracellular photosensitising drug caused little effect on the cytotoxic action of either drug (data not shown). The effect of incubation time following illumination and AlPc treatment of cells was investigated to assess the kinetics of cell death. When the MTT assay with AlPc was performed 0, 24, 48 and 72 h after illumination, the mean IC_{50} values were 0.25, 0.037, 0.019 and 0.012 μM, respectively, indicating that cytotoxicity continued to increase up to 72 h after exposure (Fig. 3).

The subcellular localisation of AlPc, AlS_2Pc, AlS_4Pc and HpD in meningioma cells was determined by confocal laser scanning microscopy. Representative fluorescence micrographs (Fig. 4) showed markedly dissimilar distribution for the four

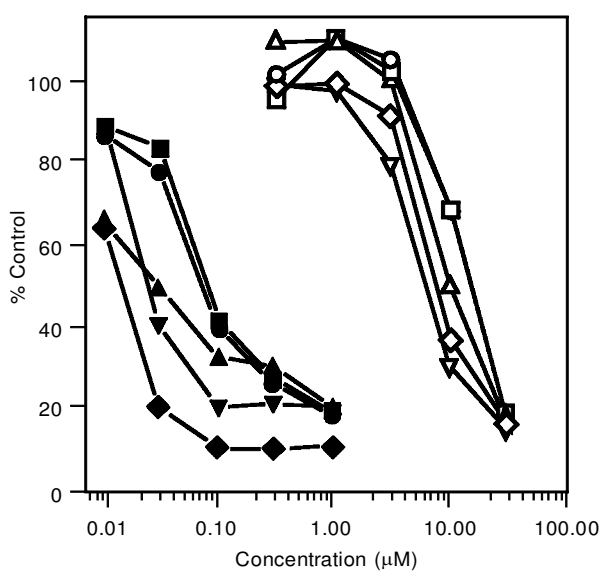


FIG. 2. Photocytotoxic effect (percentage of control) of AlPc (closed symbols) and HpD (open symbols) incubated with meningioma cells for 2 (●, ○), 4 (■, □), 8 (▲, △), 16 (▼, ;ud) and 24 (◆, ◇) h prior to illumination. Values are means from results of three meningioma cultures. No effect was observed for either of the drugs over the indicated concentration in the absence of light.

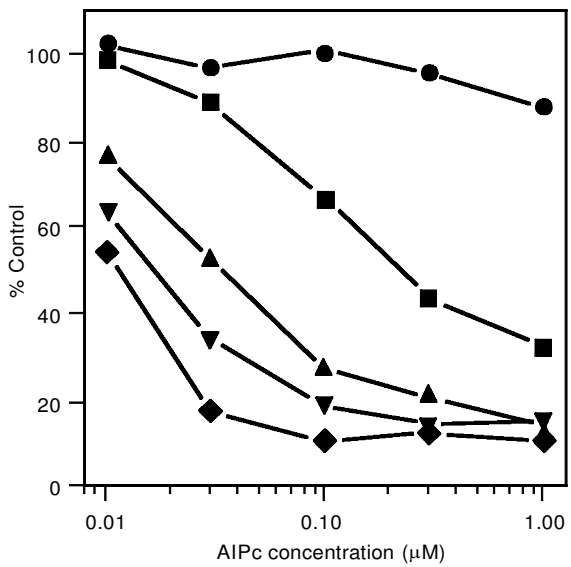
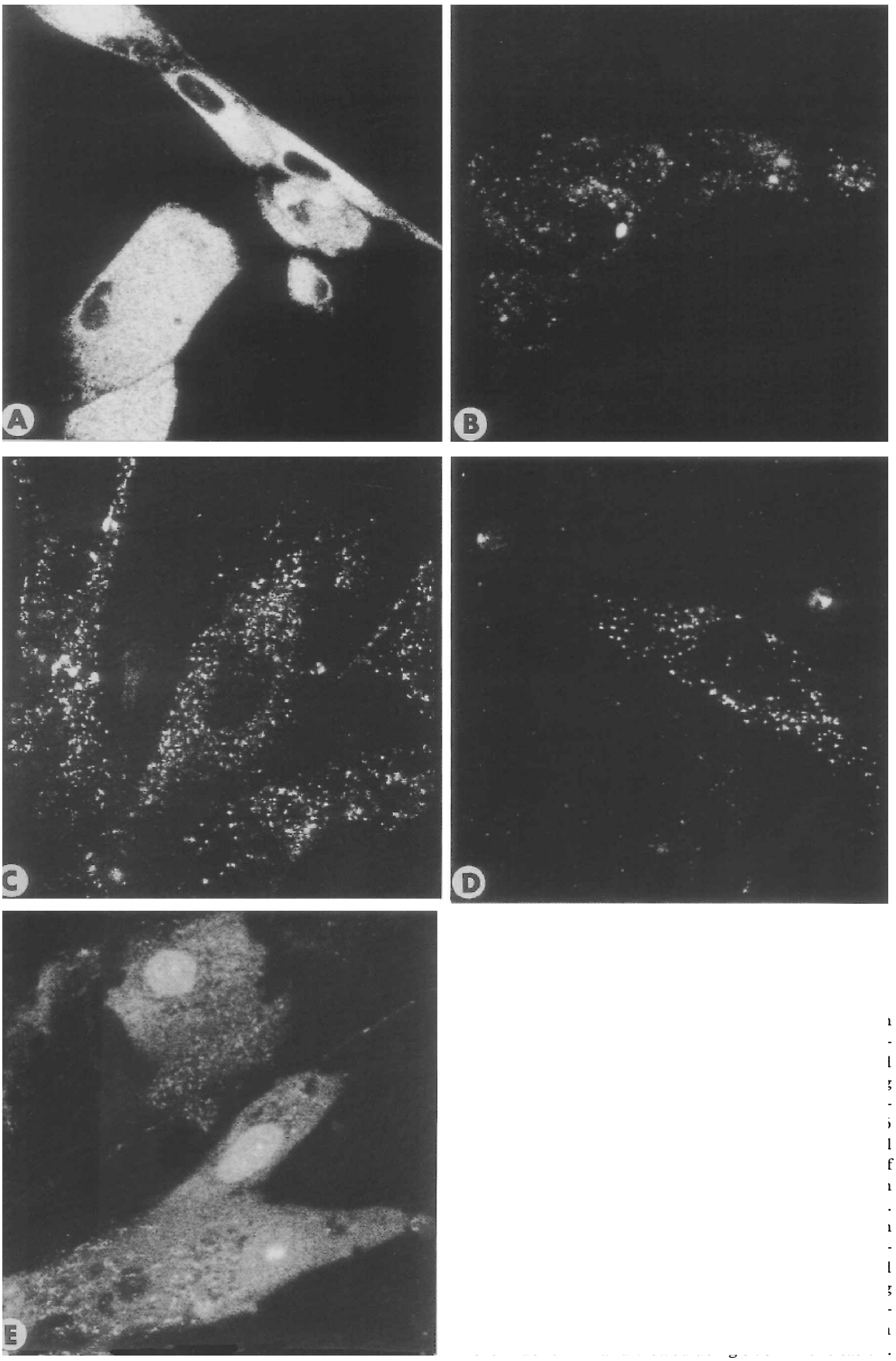


FIG. 3. Photocytotoxic effect of AlPc on meningioma cells assayed for survival 0 (■), 24 (▲), 48 (▼), or 72 (◆) h after treatment with drug illumination. Results are also shown for cultures treated with AlPc in the absence of illumination (●). Values are means of results from five meningiomas.

agents. None of the agents localized to the nucleus. The distribution of AlPc was quite diffuse through the cytoplasm (Fig. 4a). In contrast, AlS_2Pc and AlS_4Pc were localized in vesicles (Fig. 4b, 4c) which showed marked reduction in photosensitizer uptake after simultaneous incubation with ammonium chloride (50 mM, 16 h) a chemical known to increase the pH of acidic lysosomes.[23] HpD was concentrated in membranous organelles (Fig. 4d) apparently distinct from mitochondria, after comparison with cells exposed to ethidium bromide (10 μg/ml, 2 h) a known mitochondrial-localizing drug[24] (Fig. 4e).

Discussion

We have demonstrated that AlPc is at least two orders of magnitude more potent as an in vitro photosensitizer for meningioma cells than AlS_2Pc, AlS_4Pc or HpD (Fig. 1). In the absence of light, AlPc was not toxic at concentrations 50-fold higher than its *in vitro* photocytotoxic IC_{50} value, thus demonstrating a lack of non-specific cytotoxicity. Preincubation times of photosensitizing agents with various neoplastic cells prior to illumination range from 3 to 24 h in the literature.[12,25] In general, our experiments were performed with a 16-h preincubation time, but results depicted in Fig. 2 show that AlPc required an incubation time of 8 h for 50% of the 24-h effect. In this regard, AlPc was markedly different from HpD, the effects of which were 50% complete within 2 h. It appears that HpD is able to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traverse cell membranes rapidly by diffusion[11] or an efficient carrier-mediated process such as that proposed utilizing low density lipoproteins.[18] Even though AlPc is also highly lipophilic, it appears to penetrate membranes more slowly than HpD.

With each of the agents tested, an apparently resistant subpopulation of surviving cells (typically around 20%) was observed (Figs 2 and 3). The nature of this population was investigated by removing the photosensitizer after illumination and reculturing the cells for a further week. No proliferation was apparent, indicating that the cells which retained the ability to metabolise MTT were reproductively dead (data not shown). It might be thought that the cells which survive treatment at high drug concentrations may reflect the presence of a contaminating, resistant cell type such as fibroblasts. However, we have demonstrated that fibroblasts have the same sensitivity to HpD- and AlPc-PDT as meningioma cells (data not shown), a finding supported by other researchers.[11]

When meningioma cells were treated with AlPc, cytotoxicity increased with time up to 72 h following PDT (Fig. 3). When malignant glioma cells are treated with HpD, cell death increases with time up to 48 h, after which regrowth occurs.[25] Regrowth of benign meningioma cells would be unlikely to be detected *in vitro* because of their presumed inherently slow growth rate.

One of the main conclusions of this study has concerned the differences among the drug distribution of the four agents as observed by confocal laser scanning microscopy (Fig. 4). It has been suggested that the phthalocyanines are taken up by endocytosis and localize in lysosomes, while HpD enters the cell primarily by diffusion and is localized in organelles, especially mitochondria.[11] Our results indicate that the subcellular distribution of AlPc differs markedly from that of its sulphonated derivatives. It can be postulated that AlPc, which is quite lipophilic, is able to diffuse directly into the cytoplasm[26] while AlS_2Pc and AlS_4Pc are taken up by endocytosis into lysosomes. The effects of ammonium chloride, which appears to decrease the uptake of the sulphonated derivatives but not that of AlPc or HpD, are consistent with this hypothesis. The diffuse distribution of AlPc may explain its photocytotoxic potency when compared with that of the lysosomally bound sulphonated forms. Concentration in lysosomes would promote drug aggregation, which has been reported to be inversely proportional to the agent's activity.[27] Thus, while the addition of sulphonate groups render AlPc derivatives more water soluble, it may also reduce their photocytotoxic properties.

Our confocal microscopy results on HpD are consistent with the diffusion of this drug into cells and localisation in membranous organelles. However, comparison with ethidium, which concentrates in mitochondria[24] and which clearly labelled mitochondria by confocal microscopy (Fig. 4e) showed a different distribution from that of HpD. Our results are therefore consistent with other findings[14] that the fluorescence patterns of HpD do not show preferential localisation in mitochondria. It is notable that the distribution of HpD is also distinct from that of the sulphonated AlPc derivatives.

In conclusion, the results indicate unique features of AlPc, in comparison not only with HpD, but also with sulphonated AlPc derivatives. The main differences, when studied in meningioma cultures, are the high photocytotoxic potency and the subcellular distribution of AlPc. Further studies on the in vivo action of this agent are warranted.

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