Determination of the activation spectrum of aluminium phthalocyanine chloride against cultured meningioma cells using a tunable laser

Andrew C. Wilson MSc*
Gregory M. Malham MB ChB†
Raewyn J. Thomsen NZCS‡
John D. Harvey PhD*
Bruce C. Baguley PhD§

*Department of Physics, University of Auckland, †Department of Neurosurgery, Auckland Hospital, ‡Department of Pathology and §Cancer Research Laboratory, University of Auckland School of Medicine, Auckland, New Zealand.

The activation spectrum of the photosensitising agent aluminium phthalocyanine chloride (ClAlPc) was determined using cultured meningioma cells as the biological target. Cells were irradiated with tunable lasers over the wavelength range 605–1000 nm and photocytotoxicity was measured 24 h later by a tetrazolium assay. The photoactivation spectrum was found to be relatively narrow with a sharply defined maximum at 675–680 nm, a readily accessible wavelength for commercially available diode lasers. The absorption spectrum of ClAlPc in aqueous solution was broad and featureless, contrasting markedly with the activation spectrum. However, as the content of an organic solvent (dimethylsulphoxide) was increased, the absorption spectrum progressively resembled the activation spectrum. It is concluded that the absorption spectrum of ClAlPc in aqueous solution is determined by drug aggregation, which can be prevented by an organic environment. This implies that the active form of ClAlPc in cells is the monomeric species probably located in cellular membranes.

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Introduction

Photodynamic therapy (PDT) is a promising new tumour treatment modality where cell death is initiated by the photoactivation of a tumour localising photosensitising agent. The drug is inert until illuminated by light of an appropriate wavelength. Absorption of a photon by the agent results in the promotion of an electron from the ground state to an excited state, which can either decay back to the ground state or promote photoinduced chemical reactions. In the phthalocyanine system the excited singlet state can undergo intersystem crossing to the excited triplet state which can undergo either a type I electron reaction to produce free radical species, or a type II energy transfer process to produce singlet oxygen species.[1] The main objective of PDT is to induce selective tumour cytotoxicity with minimal systemic side effects.[2]

Phthalocyanine dyes offer considerable potential as sensitisers for PDT both in vitro and in vivo.[3] Several derivatives, which differ in their degree of sulphonation from the unsulphonated to the tetrasulphonated forms, have been investigated. While in vivo the photodynamic efficacy correlates positively with the degree of sulphonation, the situation in vitro is the reverse with the unsulphonated derivative being the most potent photocytotoxin.[4] An aluminium-chelated phthalocyanine derivative, aluminium phthalocyanine chloride (ClAlPc) is a convenient agent for in vitro studies on PDT and has the advantage of being easily synthesised, inexpensive, commercially available, composed of a single molecular species, and highly cytotoxic against cultured cells.[4] ClAlPc, which we have found to be soluble in aqueous solution to at least 10 μM, is cytotoxic to cultured cells at submicromolar concentrations.[5] In a previous study we have demonstrated detectable killing of meningioma cells by ClAlPc at a concentration of 10 nM[6].

Lasers provide the optimal source of photoirradiation, being highly intense, monochromatic and efficiently transmitted by an optical fibre.[7,8] Laser diodes, which are compact and economical, are particularly suitable for the treatment of solid tumors, and the long wavelengths available (600–1200 nm) offer excellent tissue penetration.[9] However, since only certain wavelength bands are available, it is important to choose a photodynamic dye which is optimally activated within this range.

Meningiomas are the most frequently occurring benign CNS tumour.[10] They are potentially suitable targets for PDT since surgical removal is not always

possible [11,12] and the available adjuvant therapies are suboptimal.[13,14] In a previous study we have used broad band red light to demonstrate the photocytotoxicity of ClAlPc against cultured meningioma cells.[6] In this study we have the same biological target, in conjunction with tunable lasers, to determine the activation spectrum of ClAlPc. We have also determined the light intensity required for ClAlPc cytotoxicity.

Materials and methods

Photosensitising agents

ClAlPc was obtained from Eastman Kodak Company (Rochester, NY), and 5 mM stock solutions, prepared by dissolution in dimethylsulphoxide (DMSO), were stored at -20°C in the absence of light.

Cell culture

Meningiomas removed neurosurgically from two 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 (μ-MEM; Gibco, New Zealand) containing 20% heat inactivated fetal calf serum (FCS; Gibco), penicillin (100 U/ml; Sigma) and streptomycin (100 mg/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.[15] For experiments, the cells were trypsinised, resuspended in fresh medium, washed and plated into 96 well trays at 10^4 cells per well (150 ml/well) within one to two passages from explanting. Cultures were incubated for 24 h, after which the medium was aspirated and 150 ml of phenol red-free medium (a-MEM; Gibco) containing FCS (20%) and ClAlPc (0.02 mM) were added to each well. Each plate contained wells to which the photosensitising agent was not added to serve as controls. Cells were incubated with ClAlPc for 16 h prior to photoirradiation.

Determination of absorption spectrum

ClAlPc was dissolved either in water, in DMSO or in 10%, 20% or 50% DMSO in water (v/v). A spectrophotometer (Shimadzu model UV3101PC) was used to obtain absorption spectra at wavelengths ranging from the ultraviolet to the near infrared. The spectral resolution of the spectrophotometer was 0.1 nm.

Determination of activation spectrum

The optimal activation wavelength of ClAlPc was determined using tunable lasers. The laser output beam was coupled into a 1 m long fibre optic bundle with the emerging cone of light directed at the sample microculture plate. The surviving cell population of each plate was assessed by a cytotoxicity assay (see below). The wavelength range 600–700 nm (red part of spectrum) utilised a CW dye laser (Coherent model 599) running on DCM laser dye (Lambda Physik LC6500), which was optically pumped by an argon ion laser (Coherent model Innova 100-15). The wavelength range 700–1000 nm was obtained using a titanium doped sapphire laser (Spectra-Physics model 3900) optically pumped by the same argon ion laser. The output power of the CW dye laser and of the titanium doped sapphire laser were both approximately 1 W over the respective tuning ranges. Wavelength tuning of both lasers was achieved using a three plate birefringent filter and wavelength measurements were made with an optical multichannel analyser (EG&G Parc model 1453A) with a spectral resolution of 0.5 nm.

The cells were exposed to the central region of the beam exiting the fibre bundle, where an intensity of 140 mW/cm^2 was measured (using an Ophir model 30A laser power meter) at the microculture plate containing the cells to be irradiated. Only the central region of the light cone exiting the bundle was used to ensure that the intensity varied by no more than 10% over the wells in use.

Cytotoxicity assay

After photoirradiation the medium was aspirated and fresh μ-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.[16,17] MTT was dissolved in PBS at 5 mg/ml, filter-sterilised and stored at -20°C in the absence of light. MTT solution was added to each well (15 ml/well) 24 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 ml/well). Control groups received identical concentrations of the photosensitising 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.

Dosage dependence

The intensity of photoillumination to which the meningioma cells treated with ClAlPc were exposed, was varied by attenuating the light beam between the laser and the fibre optic bundle using an aluminium coated glass slide of varying optical density.

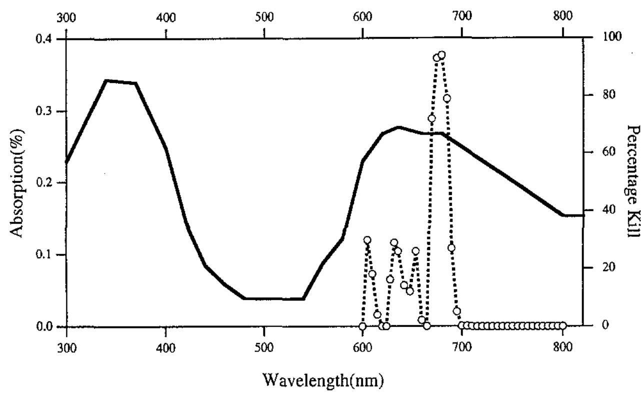

Fig. 1 Activation spectrum of ClAlPc at a concentration of 0.02 mM, as measured by photocytotoxicity of cultured meningioma cells (dashed line; right axis). The absorption spectrum of ClAlPc in aqueous solution (10 mM) is shown by the solid line (left axis).

Results

The activation spectrum of ClAlPc was determined using tunable laser sources. Previous experiments using broad band red light had determined the optimal concentration of ClAlPc to be 0.02 mM, which was the IC_{50} value (concentration required for 50% killing of cells). It should be noted that this concentration is more than two orders of magnitude less than that commonly used with sulphonated ClAlPc derivatives.[6] Other experiments had demonstrated that a concentration of ClAlPc of 0.02 mM had no effect on the viability of cells in the absence of light, and that the cells were not sensitive to light in the absence of ClAlPc.[6] Meningioma cells were exposed to laser light in the presence of 0.02 mM ClAlPc for 1 min at defined wavelengths, at 5 nm intervals, over the range 600–860 nm. Cells were then grown for a further 24 h and the surviving cells measured using the MTT assay. The activation spectrum for ClAlPc exhibited a sharply defined maximum between 675 nm and 680 nm, together with minor peaks of activation (30% cytotoxicity) at 610 nm, 640 nm and 655 nm, and no significant cell kill at wavelengths greater than 700 nm (Fig. 1).

The absorption spectrum for ClAlPc in aqueous solution over wavelengths 600–800 nm showed a broad, relatively featureless pattern (Figs. 1 & 2). As the content of DMSO was increased, the absorption spectrum progressively resembled the activation spectrum with peaks appearing at 612 and 680 nm (Fig. 2, Table 1).

The effect of varying exposure time to photoillumination of the cell sample was also investigated. Meningioma cells were exposed to laser light at a wavelength of 670 nm for 10, 20, 30, 40, 50, 60, 90, 120, 180 and 300 seconds. Cells were then cultured and assayed as before. Exposure for less than 35 seconds produced

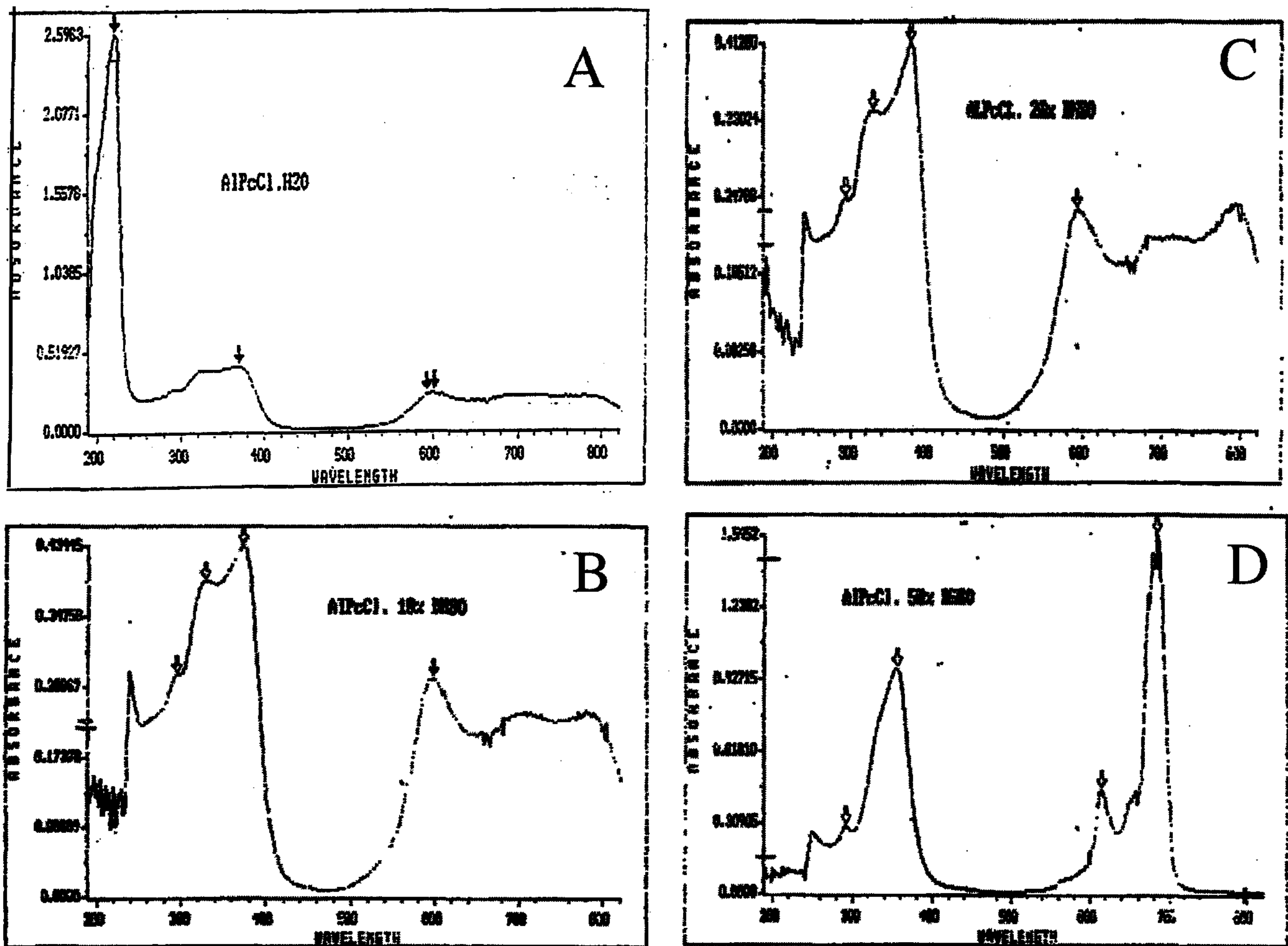

Fig. 2 The absorption spectrum of ClAlPc (10 mM) in aqueous solution (A), 10% aqueous DMSO (B), 20% aqueous DMSO (C), and 50% aqueous DMSO (D). The spectrum in 100% DMSO was similar to (D) above.

Table 1 Absorption of ClAlPc (10 mM) in the DMSO-water mixtures

Solvent	Absorbance at 612 nm	Absorbance at 680 nm	Absorbance at 800 nm
Water	0.25	0.24	0.24
DMSO 10% in water	0.24	0.22	0.21
DMSO 20% in water	0.20	0.20	0.23
DMSO 50% in water	0.45	1.53	0.01
DMSO 100%	0.40	1.37	0.00

negligible cell kill, while exposure for longer than 120 seconds caused no further cell killing (Fig. 3). Although 670 nm is not at the peak of the activation spectrum, this wavelength was used as it corresponds to that of readily available laser diodes.

The effect of variation of intensity of exposure was also measured at a constant photoillumination time of 1 min. Exposure at an intensity of less than 70 mW/cm^2 produced negligible cell kill, while exposures above 85 mW/cm^2 caused only a small further increase in cell killing (Fig. 4).

Discussion

A tunable laser system has been utilised to provide an accurate activation spectrum of ClAlPc for cultured meningioma cells. Its optimal activating wavelength was approximately 675 nm, similar to that reported for cultured V79 Chinese hamster cells.[18] This wavelength provides a distinct advantage in that it is readily accessible by diode lasers. The latter are easily obtainable, available at high power with good reliability, and provide excellent tissue penetration.[9] ClAlPc is a highly potent photosensitiser of meningioma cells,[6] is easily synthesised, inexpensive, commercially available and composed of a single molecular species. Its low aqueous solubility has made it unattractive as a potential clinical photosensitiser, but the present results suggest on the basis of its high potency that it might usefully be re-evaluated, and should provide an improved and more defined methodology for PDT research.

The results demonstrate that the activation spectrum of ClAlPc in solution does not correspond to the absorption spectrum in aqueous solution, but does correspond to that in DMSO-water (50% v/v) or in pure DMSO (Fig. 2, Table 1). It is likely that the absorption spectrum of ClAlPc in aqueous solution is broadened by drug aggregation, giving rise to the spectral changes reported in Table 1. This drug aggregation can be prevented by an organic environment.[19] The results are consistent with other studies which indicate that aggregated forms of the phthalocyanine dyes are photodynamically inactive.[20] Our previous study, employing confocal laser scanning microscopy, has shown that ClAlPc is taken up rather slowly by meningioma cells, and that cell-associated ClAlPc is diffusely distributed throughout the cytoplasm. This behaviour contrasts with that of sulphonated ClAlPc derivatives, which are concentrated in lysosomes.[6] The much greater in vitro potency of ClAlPc in comparison to the less lipophilic sulphonated ClAlPc derivatives suggests that the active form of ClAlPc in cells is the monomeric species dissolved in cellular membranes. This is the form thought to provide the highest yield of singlet oxygen, which is proposed to be the dominant cytotoxic species mediating photodynamic therapy.[19]

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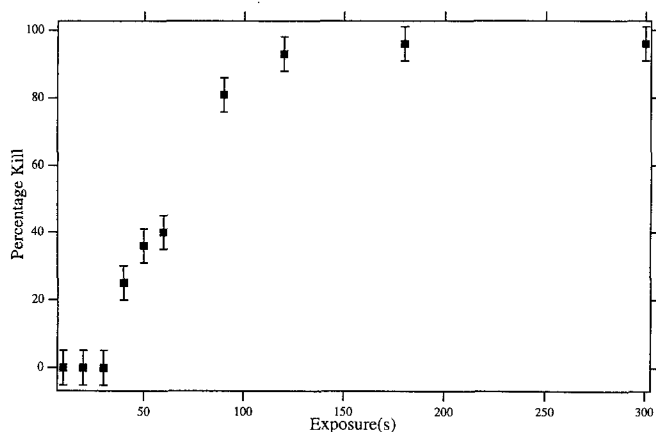

Fig. 3 Dependence of photocytotoxicity of ClAlPc (0.02 mM) on exposure time, using an illumination wavelength of 670 nm and an exposure intensity of 140 mW/cm^2. Vertical bars represent estimated standard errors of determinations.

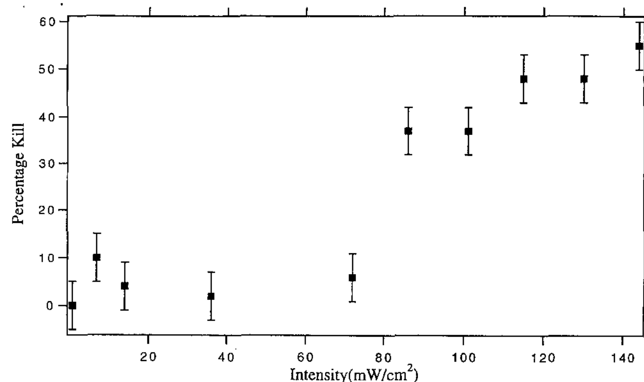

Fig. 4 Dependence of photocytotoxicity of ClAlPc (0.02 mM) on exposure intensity using an illumination wavelength of 670 nm and an exposure time of 1 minute. Vertical bars represent estimated standard errors of determinations.

Correspondence and requests for offprints:
Dr Gregory M Malham
Department of Neurosurgery
Auckland Hospital
Private Bag 92024
Auckland
New Zealand
Tel:(64-9) 379 7440 extn. 7839
Fax:(64-9) 307 4924

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